A multidisciplinary study of human exposure to arsenic and other trace elements

CLAUDIA CASCIO

Thesis submitted in part fulfilment
of the requirements of the Award of

Doctor of Philosophy
De Montfort University

Sponsor of the Research: Marie Curie Actions

July 2011

To my family

"Imagination is more important than knowledge"

Albert Einstein

Acknowledgments

There are not sufficient words to thank my first supervisor Dr. Parvez Haris for giving me the chance to attend the PhD course under his excellent supervision. He has taught me principles and rules of research and most importantly he showed me with enthusiasm that perseverance, passion, honesty and creativity are the most important qualities to reach high targets in research and in life.

I want to sincerely thank my second supervisor Prof. Richard Jenkins for the high quality of his work and suggestions and for constantly helping me in reaching and pursuing my objectives with professionalism.

My thanks also go to all the volunteers from UK, Italy (and especially the Italian Association for Multiple Sclerosis of Linguaglossa) and Bangladesh that contributed to this study by providing biological specimen and information and without whom this entire research could not be possible.

This project was supported by the AquaTRAIN MRTN funded under the European Commission Sixth Framework Programme (2002-2006), Marie Curie Actions – Human Resources and Mobility Activity Area, Research Training Networks (Contract No. MRTN-CT-2006 035420). I would like to thank Prof. David Polya of Manchester University for proficiently coordinating the project and all the other scientists of the network for providing suggestions and support. I also want to acknowledge colleagues and friends from the Aquatrain project that shared this 3 years of adventure, I want to especially thank Barbara Casentini (forever roommate!) and neo Dr. Julia Leventon.

I would like to thank my colleagues at De Montfort University of Leicester that several times helped me in practical and scientific aspects of my life and especially Dr. Antonio Signes-Pastor, Shaban Al Rmalli, Essam Talha and my dear friend Jing Pan.

I am extremely grateful to Prof. Andy Meharg and Prof. Joerg Feldmann of the University of Aberdeen for hosting me in their laboratories and teaching me a lot on arsenic in the environment and humans. A special thanks goes Dr. Andrea Raab for her help and patience.

I want to thank Prof. Ahsan of the Chicago University for providing urine samples from Bangladeshi volunteers and Dr. Argos for helping with data treatment.
I also want to thank Dr. Michael Watts of the British Geological Survey and Dr. Sergio Calabrese of the University of Palermo.

I would like to thank and acknowledge Dr. Luca Montanarella and the Join Research Centre of the European Commission for hosting in his research group. I would like to thank Dr. Luis Rodriguez-Lado for his excellent supervision and for the constant contribution and support he gave to my project and decisions.

I am particularly thankful to Prof. Alessandra Nicoletti of the Department of Neurosciences of University of Catania for the proficient collaboration we could establish on multiple sclerosis and trace elements. I am also grateful to Prof. Zappia, Prof. Patti, Salvo Lo Fermo, Elisa Bruno, Silvia Messina and Graziella Quattrocchi and to all the people who collaborated.

I am grateful to all the people I have met in 3 years of travelling up and down Europe to encourage me including Anne-Marie Carey, Raif Yucel, Melinda Urzì, Motje Wolf and Jackie Calderwood.

I would like to thank Nello, for supporting and tolerating my PhD mode in the last months spent in Catania.

Finally, from the deep of my heart I thank my mother and my father who told me to always aim for the best I can, and that constantly encouraged and supported all my decisions with love.

ABSTRACT

Arsenic (As) is a carcinogenic agent that is present in varying levels in environmental matrices including water and food. Long term As exposure can lead to skin lesions, peripheral neuropathy, diabetes, renal system effects and cardiovascular diseases. Bio-monitoring of human urine, toenail, serum and cerebrospinal fluid was carried out in this thesis to assess the exposure to arsenic and other trace elements. A multidisciplinary approach based on Inductively Coupled Plasma Mass Spectrometry (ICP-MS), HPLC-ICP-MS and Proton-Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR) in conjunction with a questionnaire based survey was employed.

The impact of rice consumption (a well-known vector of arsenic in the general population) on human urinary As levels was assessed. Results obtained show that the Bangladeshi (UK-B) community in the United Kingdom, who consume ca. 30-fold more rice than the white Caucasians (UK-C), are exposed to a higher level of arsenic. ICP-MS and HPLC-ICP-MS revealed a significant increase in dimethylarsinic acid (DMA) and inorganic arsenic (iAs) species in UK-B compared to UK-C, while cationic compounds were lower in UK-B than in UK-C. DMA and iAs levels in the Bangladeshis were positively correlated to rice consumption. Rice is likely to be responsible for the increase in levels of DMA and iAs in urine of UK-B. The link between this and the disproportional occurrence of diabetes and cardiovascular diseases (CVD) in UK-B needs to be investigated. Another important finding of this study is that the DMA to monomethyl arsenic (MA) ratio, which is often used as an indication of arsenic methylation capacity, should be applied with caution in populations consuming large quantities of rice because variation in the quantity and type of rice eaten may alter the urinary DMA levels and thereby the DMA/MA ratio.

Urinary arsenic, selenium, copper and zinc were monitored for a group of Bangladeshis, Pakistanis, Indians and Caucasians living in the UK. The most striking finding was the increase in urinary copper in the UK-B group compared to other ethnicities and to reference values reported for the general UK population. Among the possible reasons for this could include dietary exposure via ethnic food consumption or a change in copper metabolism in the Bangladeshis. High serum copper levels have been correlated to CVD in the US population. In this context, further work is recommended to investigate if there is a

relationship between urinary copper and the disproportionately high incidence of CVD in UK Bangladeshis.

An approach based on ^{1}H-NMR was used to detect changes in human urinary metabolomic profile as a function of As exposure through different routes. For this, the urine of UK-B, UK-C and a group residing in Bangladesh (BD-B) were monitored. The effects of other factors were explored, including arsenic urinary profile, chewing pan, ethnicity, rice consumption, selenium and diabetes. The three populations show distinctive metabolomic profiles. Urinary arsenic speciation was used in evaluating the effects of arsenic on the metabolomic profile for the UK group. This revealed that the %DMA positively correlates to %N,N-dymethylglycine, %alanine and %betaine. Comparative analysis of the ^{1}H NMR spectra revealed that the BD-B urinary profiles were depleted in the number and quantity of metabolites. Visible signs of lower protein intake and undernourishment emerged from the urinary metabolomic profile of BD-B including a 2.5 decrease in creatinine levels compared to UK-B. Urinary creatinine and the metabolomic profile provide evidence for undernourishment in the BD-B population group that was not evident from previous studies on dietary protein intake in this population performed using food frequency questionnaires. Public health officials might consider also using bio-monitoring studies for nutrient intake rather than solely relying on estimations from food frequency questionnaires. The results reveal the complexity of the subject and pave the way for future studies, highlighting the need for awareness about diet and other specific confounding factors.

Multiple Sclerosis (MS) is considered a multifactorial disease and its cause remains unknown. A case-control study on a MS cluster from the volcanic region of Mt. Etna (a natural emitter of geogenic trace elements in the environment) was undertaken. Urine and toenails were monitored for trace elements along with food consumption and life-style habits. Levels of a range of trace elements were reported for the first time for a population living in the Mt. Etna region. No significant differences were found in trace element levels in urine and toenails of MS patients and controls. However, urinary levels of nickel, manganese and selenium were higher than those reported in the literature for the general population from Italy, Germany and the UK. These findings and observations might suggest a role for nickel in the pathology of MS. However, larger studies on the possible role of nickel on MS, and trace elements in general, should be performed.

Cerebrospinal fluid (CSF) and some serum from MS patients and controls from the Mt. Etna region were also monitored in this study using ICP-MS. There were significant differences in the trace elemental profile of CSF of MS volunteers and controls, including an increase in arsenic and zinc in the CSF of MS patients. Lead, aluminium, cadmium and molybdenum were significantly increased in the CSF of MS patients as well. In contrast, selenium was lower in MS patients compared to controls. The enrichment of certain trace elements in the CSF of MS patients could be the result of an impairment of the blood brain barrier and tight junction disruption due to MS and its progression, resulting in serum protein leakage and trace elements across the blood–brain barrier. Studies are necessary in the future to identify the chemical species present in the CSF and also determine their role in biological processes including their harmful effects on the brain.

Published Papers from this work:

I. C. Cascio, A. Raab, R. O. Jenkins, J. Feldmann, A. A. Meharg and P. I. Haris (2011) **The impact of a rice based diet on urinary arsenic** - Journal of Environmental Monitoring (DOI: 10.1039/c0em00482k)

II. C. Cascio, L. Rodriguez-Lado, D.A. Polya, M. Zappia, F. Patti, A. Nicoletti, R.O. Jenkins and P.I. Haris (2009) **Geogenic trace elements in groundwaters in the Mt Etna region: Geostastistical, proteomic and epidemiological approaches to assessing human exposure and health risks**- Goldschmidt Abstracts 2009 - Geochimica et Cosmochimica Acta, 73(13), A181-A254 Awards Ceremony Speeches and Abstracts of the 19th Annual V.M. Goldschmidt Conference.

Abstracts presented in Conferences and Workshops:

I. C. Cascio, A. Raab, R.O. Jenkins, J. Feldmann, A. Meharg, P.I. Haris (2010) **Biomonitoring of arsenic exposure in a UK Bangladeshi population**, AquaTRAIN International Conference Orleans, France July 2010

II. C. Cascio, R. O. Jenkins and P. I. Haris (2010) **Looking for changes in urinary proteomics and metabolomics profiles as a function of arsenic exposure**, AquaTRAIN International Conference Orleans, France July 2010

III. C. Cascio, A. Nicoletti, S. Lo Fermo, M. Zappia, E. Bruno, L. Rodriguez-Lado, R. O. Jenkins, P.I. Haris (2010) **Towards the identification biomarkers for multiple sclerosis in human urine using ^{1}H-NMR Spectroscopy**, Metabolomics 2010 Amsterdam, Netherlands June 2010

IV. C. Cascio, L. Rodriguez Lado, A. Nicoletti, M. Zappia, F. Patti, D. Polya, P. Lyhgoe, R. O. Jenkins, L. Montanarella, P. I. Haris (2008) **Volcanism and environmental health: a multidisciplinary approach applied to the Mt. Etna region**. 3rd Central and Eastern Europe Conference on Health and the Environment, Cluj-Napoca, Romania, October 2008

V. C. Cascio, A. Nicoletti, E. Bruno, S. Lo Fermo, S. Messina, L. Rodriguez-Lado, D. Polya, P. Lythgoe, R.O. Jenkins, P. I. Haris (2008) **A holistic approach for the study of Multiple Sclerosis in the Mount Etna region.** 2nd PRAMA Workshop on Risk assessment, Manchester, UK, June 2008

VI. C. Cascio, R.O. Jenkins, E.I. Brima, D. Polya, P.I. Haris (2008) **Understanding arsenic metabolism in humans through proteomics and metabolomics**. 2nd PRAMA Workshop on Risk assessment, Manchester, UK, June 2008.

Table of Contents

Chapter 1
INTRODUCTION AND BACKGROUND

More than 140 million people in at least 70 countries worldwide are affected by arsenic in ground and surface water (Ravenscroft, 2007). Arsenic, in its inorganic form, is a class one carcinogenic agent (IARC, 2004) responsible for cancer of the lungs, bladder and skin. Because of certain geogenic or anthropogenic processes, arsenic and other toxic or essential trace elements start their biogeochemical cycle and become available for humans through different exposure routes. The bio-monitoring activity of human biofluids to assess levels of exposure to trace elements is important to determine associations with morbidity and mortality, identify the amounts which are required to sustain a healthy state, identify nutritional status and for the implementation of government regulations (Hamilton *et al.*, 1994a). The relationship between chemicals in the environment and human health is illustrated in Figure 1-1 using arsenic as an example.

This thesis presents the outcome of bio-monitoring studies carried out to assess human exposure to trace elements (including arsenic, selenium, zinc, copper and nickel) and involvement in pathological outcomes; a multidisciplinary approach and a range of spectroscopic techniques were used to achieve these goals (see section 17 of this Chapter).

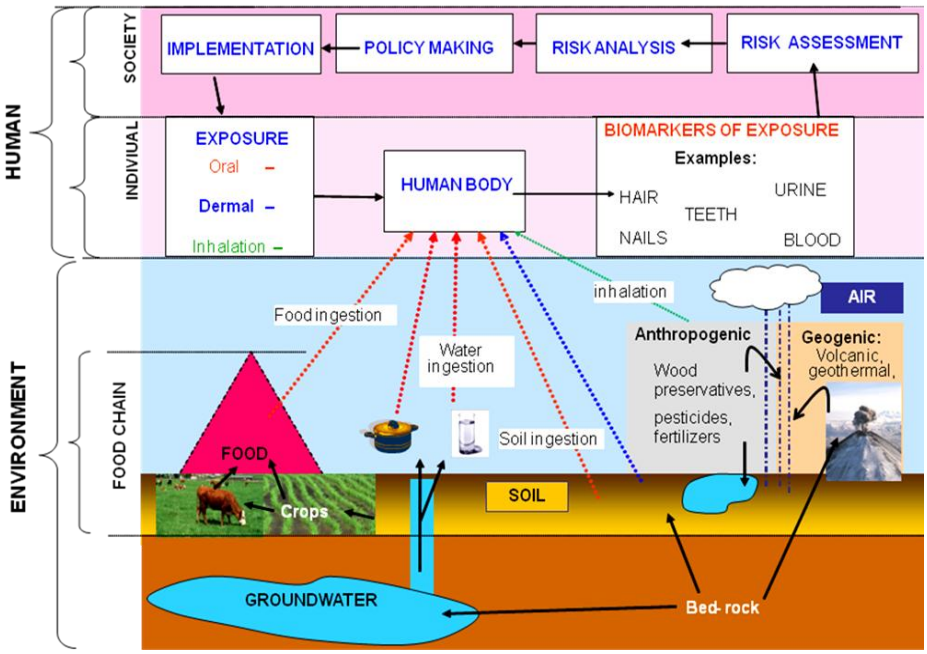


Figure 1-1: multiple sources of arsenic exposure for humans: pathways, monitoring and decision making.

1. Trace elements and health

Trace elements are present in the environment (in soil, air, water) as a result of geogenic mobilization processes such as rock weathering, volcanic emissions and geothermal phenomena (Hansell *et al.*, 2006) and anthropogenic processes including mining, farming, burning, and industrial activities. In addition, trace elements are constituents of food and are normally present in drinking water. In most geochemical systems, 99% of the mass can be accounted for by just eleven elements. (H, C, O, N, Na, Mg, P, S, Cl, K, Ca) as highlighted in the periodic table in Figure 1-2. The remaining elements can be considered as 'trace' elements occurring in parts per billion (ppb) and parts per million (ppm). Goldschmidt's classification divides elements according to geochemical affinity in lithophile, calchophile, siderophile and atmophile (Goldschmidt, 1923). More recently, the addition of the new biophile category was proposed (Hollabaugh, 2007). From a biological point of view, trace elements can be also defined as shown in Figure 1-2 (Lindh, 2005).

The World Health Organisation (WHO) (WHO, 1996) defines trace elements as those elements present in a matrix at a concentration lower that 250 µg/g. WHO divides trace

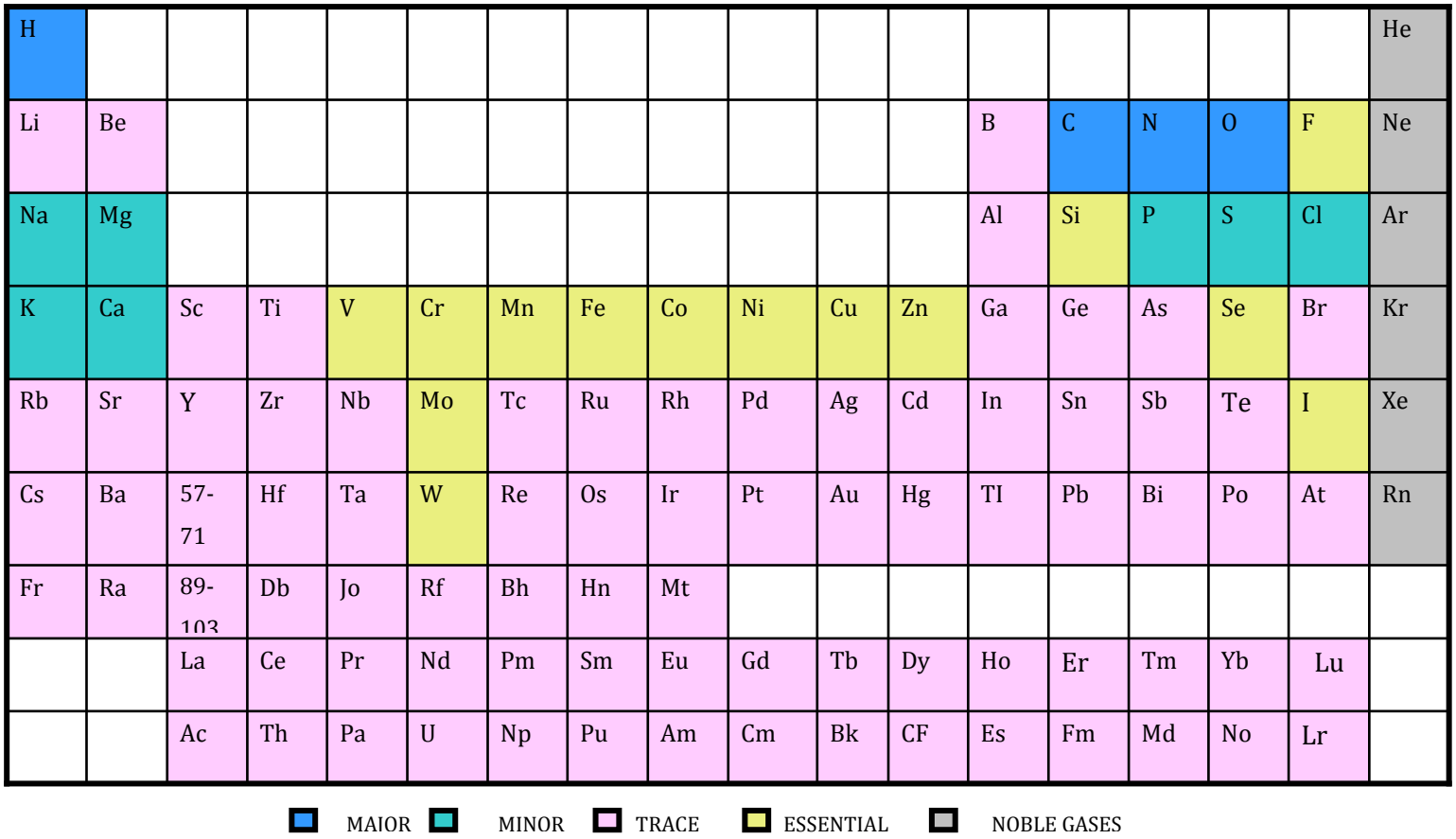


Figure 1-2: Major, minor and trace elements essential for humans, modified from (Lindh, 2005)

elements in human nutrition as: (i) essential, (ii) probably essential and (iii) potentially toxic as in Table 1-1. WHO defines essential for humans, elements for which a deficit in exposure causes a reduction of a particular beneficial physiochemical process.

Finally, the International Union of Pure and Applied Chemistry (IUPAC) defines trace elements as "any element having an average concentration of less than about 100 parts per million atoms (ppma) or less than 100 μg/g"(IUPAC, 1997).

Table 1-1 Essential and not essential trace elements in human nutrition (WHO, 2006)

Classification	**Element**
Essential	I, Zn, Se, Cu, Mo, Cr
Possibly essential	Mn, Si, Ni, B, V
Potentially toxic elements with a function	F, Pb, Cd, Hg, As, Al, Li, Sn

2. Chemistry of arsenic

Arsenic (As) is a metalloid belonging to the 15th group of the periodic table (see Figure 1-2). Elemental arsenic As(0) is insoluble in water while arsenic salts exhibit a large range of solubility depending on pH and Eh. Arsenic can exist in four valence states (−3, 0, +3 and +5). Arsenite (As(III)) is dominant under reducing conditions, while the pentavalent form, arsenate (As(V)) is generally the more stable form in oxygenated environments (WHO, 2001). At 20°C and 1 atm pressure, As is a grey, brittle solid. As can form minerals with S, some of them are shown in Figure 1-3.

Arsenic is the 20th most common element in the earth's crust and is a component of more than 245 minerals (Mandal and Suzuki, 2002). Arsenic compounds can be found in rock, soil, water and air as well as in plant and animal tissues; more than 20 compounds of arsenic (some reported in Figure 1-4) exist in the environment with considerable differences in toxicity and stability. Table 1-4 provides details about nomenclature of main arsenic compounds.

The degree of instability of the main species is MA(III) > DMA(III) > As(III) > As(V) > MA(V) > DMA (V)>AB (Gong *et al.*, 2001).

Table 1-2: Arsenic species in the environment and in humans

Abbreviate name	**Compound name**
As (III)	Arsenite
As(V)	Arsenate
MA(III)	Monomethylarsonous acid
DMA(III)	Dimethylarsinous acid
MA(V)	Monomethylarsonic acid
DMA(V)	Dimethylarsinic acid
TMAO	Trimethylarsine oxide
TETRA	Tetramethylarsonium iodide
AB	Arsenobetaine
AC	Arsenocholine
AsSUG	Arsenosugars
AsLIP	Arsenolipids

Organic arsenic compounds such as arsenobetaine (AB) and arsenosugars are mainly found in marine organisms, mostly in marine algae and in mussels and oysters (Sanchez-Rodas *et al.*, 2002). Another class of organoarsenicals are the arsenolipids, six arsenic-containing fatty-acids that have been reported in fish oil (Rumpler *et al.*, 2008). A better detail of arsenic species found in urine is in section 3-10 of Chapter 2.

A) **B)**

Figure 1-3: Arsenic minerals (A) Realgar (AsS) and Orpiment (B). Realgar is used as medication in Traditional Chinese Medicine. Pictures from http://www.depurazioneacquearrigoni.it/arsenico/utilizzo.html

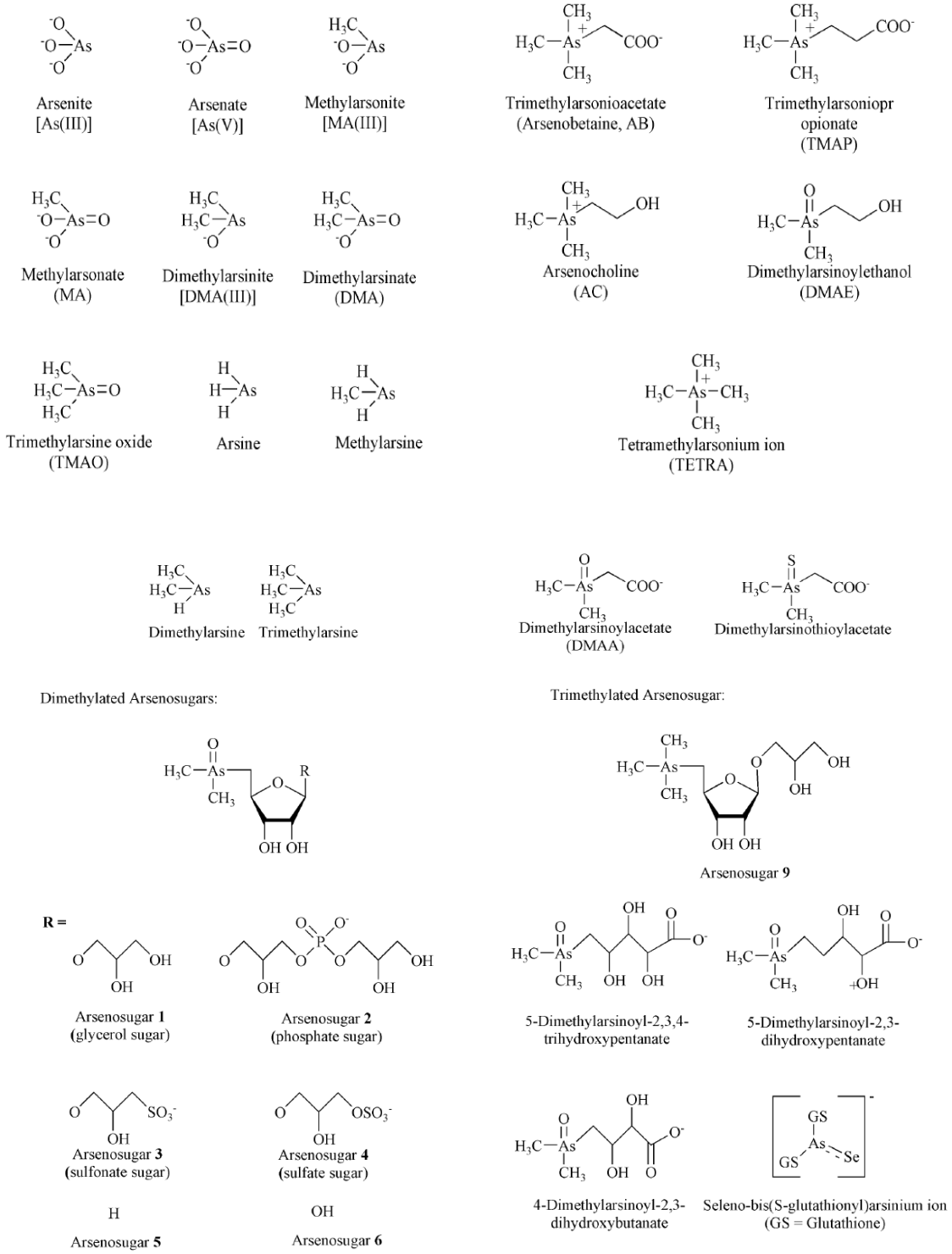


Figure 1-4: Structure and nomenclature of some arsenic species (Francesconi and Kuehnelt, 2004)

3. Arsenic occurrence in the world

Arsenic pollution of both geogenic and anthropogenic origin is reported in many sites of the world. The main sites are shown below and a map is presented in Figure 1-5 (Ravenscroft, 2007).

3.1 Europe

There are two major occurrences of alluvial arsenic pollution in Europe: the Po Basin in North of Italy, and the inland part of the Danube in Hungary, Croatia and western Romania, the so called Pannonian Basin. Significant arsenic pollution is reported in Cornwall and Devon because of a long history of mining (Kavanagh *et al.*, 1998).

Table 1-3 reports occurrence of arsenic in groundwaters of some European sites. Arsenic has been reported in groundwater of the Chalkidiki area in Greece (Kouras *et al.*, 2007); in Italy concentrations up to 6900 and 3800 ppb have been found in the Phlegrean Fields and in Ischia respectively (Aiuppa *et al.*, 2006). In central Spain, a survey of 514 well in the Duero Cenozoic Basin showed an average concentration of As is 40.8 ppb (Gómez *et al.*, 2006). Naturally occurring arsenic in groundwater of the Heubach plain, North Rhine-Westphalia, (Germany) has been reported (Banning *et al.*, 2008b, Banning *et al.*, 2008a). In Finland high contents of arsenic, fluoride and radon in drilled wells in certain regions has been found (Knutsson, 2008).

Table 1-3: Arsenic concentration in some European groundwaters

Region (Country)	Max [µg/L]	Min [µg/L]	Mean [µg/L]	Reference
Transylvania (RO)	176	0		(Gurzau and Gurzau, 2001)
Chalkidiki (GR)	1,840	1		(Kouras *et al.*, 2007)
Phlegrean fields (IT)	6,900	1.6		(Aiuppa *et al.*, 2006)
Ischia (IT)	3,800	2.6		(Aiuppa *et al.*, 2006)
Nea Triglia-Eleachoria Chalkidiki (GR)	2,825	4.6	1,128	Casentini (private communication)
Duero Basin (SP)	210	0.5	40.8	(Gómez *et al.*, 2006)
Croatia	610	10		(Habuda-Stanic *et al.*, 2007)

3.2 South and Central America

Areas affected by arsenic are reported in South America including the Chaco-Pampean Plain, where aquifers comprise Tertiary loess deposits (in the Pampean Plain) and Tertiary and Quaternary fluvial and aeolian sediments (in the Chaco Plain) in Argentina (Bundschuh *et al.*, 2004); and the geothermal sources of surface water in northern Chile (Dittmar, 2004) and Bolivia. In Central America, there is extensive arsenic pollution in Mexico (Del Razo *et al.*, 1990, Camacho *et al.*).

3.3 Africa

Based on current knowledge, Africa is the least affected continent. There is some occurrence of arsenic in the volcanic region of southwest of Cameroon (Mbotake, 2006). Moreover, Ravenscroft (Ravenscroft, 2007) predicts the largest African areas with arsenic-risk occur around the Rift Valley, Ethiopian highlands and Ruwenzoris.

3.4 Asia

Several affected areas are present in Asia, the worst affected in the continent are the Indo-Gangetic and Brahmaputra plains, but also the Mekong, Red and Irrawaddy river plains. Affected countries are Bangladesh (Nickson *et al.*, 2000), Vietnam (Berg *et al.*, 2001), Cambodia and West Bengal (India) (Charlet and Polya, 2006). Arsenic is reported in Taiwan (Tseng *et al.*, 1968a) and Japan and in the island of Sumatra in Indonesia (Winkel *et al.*, 2008). In China, the middle section of the Yellow River is known to be polluted by arsenic. Furthermore, Ravenscroft (Ravenscroft, 2007) predicted the presence of contaminated groundwater in a densely populated area where there are no, or very few, reports of pollution: the Tigris-Euphrates basin in Iraq.

3.5 USA and Canada

Arsenic pollution occurs over large areas of North America. Arsenic pollution is reported adjacent to the Rockies, in the Rio Grande Basin and in the Carson Desert in the southwest, the Willamette Basin of Oregon, the Snake River in Idaho, the Platte River in Idaho and at Fairbanks and the Kennai Peninsula in Alaska (Ravenscroft, 2007). Concentrations are greater in the Interior Plains and the Rocky Mountain System. Investigations of ground water in New England, Michigan, Minnesota, South Dakota, Oklahoma, and Wisconsin within the last decade suggest that arsenic concentrations exceeding 10 μg/L are more

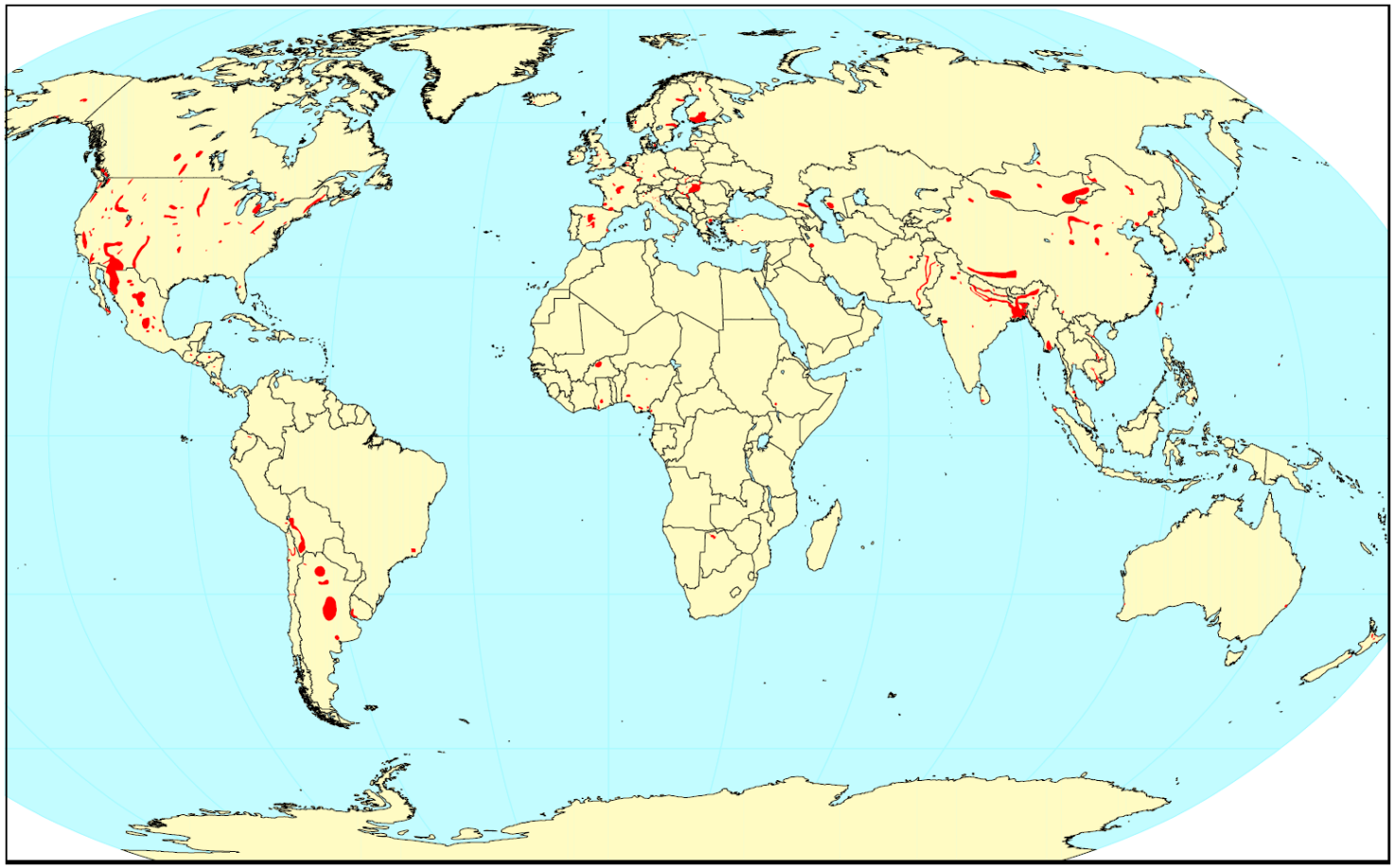


Figure1-5: arsenic in the world (Ravenscroft, 2007).

widespread and common than previously recognized (Welch *et al.*, 2000).Furthermore, anthropogenic arsenic occurs in Texas (Geraghty and Inc, 1996), Massachusetts (Davis *et al.*, 1994), Maryland (Vroblesky *et al.*, 1989) and South Dakota (Carter *et al.*, 1998).

4. Arsenic in water

The average distribution of arsenic in ground and sea water is generally 1-2 μg/L (WHO, 2001) although in areas with volcanic rocks, sulphide mineral deposits or geothermal activity it can reach up to 6,400 μg/L (Nakahara *et al.*, 1978). Arsenic species identified in water include As (III), As(V), MA(V), MA(III), DMA(V), DMA(III) and TMAO (IARC, 2004). Normally groundwater does not contain methylated forms of arsenic but lake and pond waters contain MA and DMA (Hasegawa *et al.*, 1994). The concentration of naturally occurring arsenic in groundwater varies with climate and geology. Surface and groundwaters near former mining or smelting sites can contain elevated As levels (Jones, 2007a).

Microorganisms can be involved in biogeochemical transformations influencing the solubility of arsenic in groundwaters and performing different types of reactions including the reduction of As (V) to as As(III) and the oxidation of As(III) to As(V).

As(V) is usually found in shallow waters. As concentration in groundwaters has been found to be correlated to the depth with higher values in wells > 15m deep than in shallow wells (Agusa *et al.*, 2009) (Harvey *et al.*, 2002) (Polya *et al.*, 2005).

5. Arsenic in soil

Natural soil concentrations of arsenic typically range from 0.1 to 40 mg/kg with an average of 5- 6mg/kg (WHO, 2001). Geogenic As in soil mainly derives from the transport of particulate from weathered minerals in the parental material. Arsenic mobility in soils depends, as in water, on pH and Eh. Geological and geochemical processes contribute to the transfer of As to soils. High As concentrations in soil, typically caused by anthropogenic sources (near smelting operations or older orchards where As pesticides were used) range from 10 to > 1000 mg/kg (Smith *et al.*, 1998). As residing in soil usually adsorbed onto soil particles, can be transported over short distances via leaching. Soil

absorption capacity is correlated positively to the content of Fe oxide, Mg oxide, Al_2O_3, and clay (Jones *et al.*, 2006).

Irrigation with groundwater contaminated by arsenic can enrich arsenic levels in soil. Paddy fields irrigated with arsenic-contaminated groundwater produce rice with elevated levels of arsenic in grains, and ultimately results in human exposure (Williams *et al.*, 2005a). Rice is the most widely consumed cereal in the world. It also contains the highest levels of As compared to other cereals (see specific section 7.1 Arsenic in Rice of this Chapter). This has raised concerns regarding its health impact on millions of people in Asia who rely on a staple diet of rice and especially those who also drink As contaminated water.

A map of As distribution in England (Appleton *et al.*, 2008) is presented in the Appendix (Figure A1-1). Figure A1-2 in the Appendix shows maps of trace elements in top soil (including As) as derived by mean or regression-kriging approach by Rodriguez-Lado *et al. (*Rodríguez Lado *et al.,* 2008*)* from the Forum of European Geological Surveys Geochemical database (FOREGS). In Chalkidiki Prefecture (Northern Greece) an average concentration 127 mg/kg (min: 6; max= 538 mg/kg) in soil has been found (Casentini, unpublished data).

It is crucial to note that the total amount of arsenic in soil is not *per se* an indication of the degree of toxicity of soil. The toxicity of the soil is reflected by the fraction of the total As that is bioaccessible and bioavailable for humans and can therefore be absorbed in the gastro intestinal tract and taken up by the human body, if ingested. In other words, soils with high total As may not pose a high risk for human health because the As is not in a bioavailable form. In contrast, soils with a relatively low total As concentration may be hazardous if they contain more bioavailable As due to differences in As mobility and species. Various studies have investigated the key factors in As bioavailability from soil. Physico-chemical properties have an influence on the content of As; in sandy soils As is more mobile and bio-available than in clayey soils. In addition, organic matter content, particle-size distribution (clay and sand content) and water-soluble arsenic are determining factors that significantly influence arsenic bioaccessibility (Girouard and Zagury, 2009) (Pouschat and Zagury, 2006).

In aerobic environments, As(V) is the dominant form and is strongly absorbed onto clays, iron, manganese oxides/hydroxides and organic matter (Mandal and Suzuki, 2002). On the

contrary, in reducing environments As(III) can be found. In oxidizing conditions MA have been found, derived from microbiological methylation activity (Mandal and Suzuki, 2002).

For plants, a general increase in As toxicity is reported when pH becomes lower. Phosphorous has been reported to displace As from its binding sites in soil (Azizur Rahman *et al.*, 2008).

6. Arsenic in air

The mean total arsenic concentrations at a global scale in air is estimated to range from 0.02 to 4 ng/m^3 in rural areas, from 3 to about 200 ng/m^3 in urban areas and to be >1000 ng/m^3 in the proximity of industrial sources, with a decreasing trend due to pollution abatement measures (WHO, 2001).

Atmospheric arsenic usually occurs in particulate form arising from both natural sources, such as volcanic activity or forest fires, and anthropogenic sources, such as burning of fossil fuels, automobile exhaust and tobacco smoke. In air, As is mainly absorbed on particulate with As(III) and As (V) as main forms. Organic species in air are negligible, except in areas with arsenic pesticide applications (ATSDR, 1993).

Geogenic sources of As in air, represented by volcanic emissions, have been estimated by (Signorelli, 1997) in a survey of fumarolic concentrates from different volcanoes around the world. They found the highest total As content (1.2-30 μg/g) in the Island of Vulcano, Southern Italy.

Arsenic levels in Europe range between 0.2 and 1.5 ng/m^3 in rural areas, 0.5-3 ng/m^3 in urban ones and no more than 50 ng/m^3 in industrial regions (DG Environment European Commission, 2000).

The estimated intake of As for humans from air is generally less than 1 μg (WHO, 2003).

7. Arsenic in food

Arsenic can be found in different categories of food with a high variation of concentrations and species. Therefore, as recommended by the European Food Safety Authority (EFSA), risk assessment for arsenic in food must be based on estimation of inorganic arsenic rather than just total (CONTAM, 2009). A total diet study (TDS) has been conducted for the

British population (FSA, 2003), assessing the content of both total and inorganic arsenic in main food types as reported in Table 1-4.

Fish presents the highest total arsenic content compared to other food groups (3,990 µg/kg), followed by poultry (22 µg/kg). However, considering the percentage of iAs, cereals are most affected by iAs, containing about 67% of iAs (12 µg/kg) compared to fish which contains just 0.03% (15 µg/kg) (see Figure 1-6).

Table 1-4: Arsenic content in different food types from the British TDS (FSA, 2009).

Food Group	Inorganic As [mg/kg]	Total As [mg/kg]
Bread	<0.01	0.005
Misc. Cereal	0.012	0.018
Offal	<0.01	0.008
Meat Product	<0.01	0.005
Poultry	<0.01	0.022
Fish	0.015	3.99
Oils & Fats	<0.01	<0.005
Eggs	<0.01	<0.003
Sugar & Preserves	<0.01	0.009
Green Vegetables	<0.01	0.004
Potatoes	<0.01	0.005
Other Vegetables	<0.01	0.005
Canned Vegetables	<0.01	0.001
Fresh Fruits	<0.01	0.001
Fruit Products	<0.01	0.003
Beverages	<0.01	<0.001
Milk	<0.01	<0.001
Dairy Products	<0.01	<0.003
Nuts	<0.01	0.007

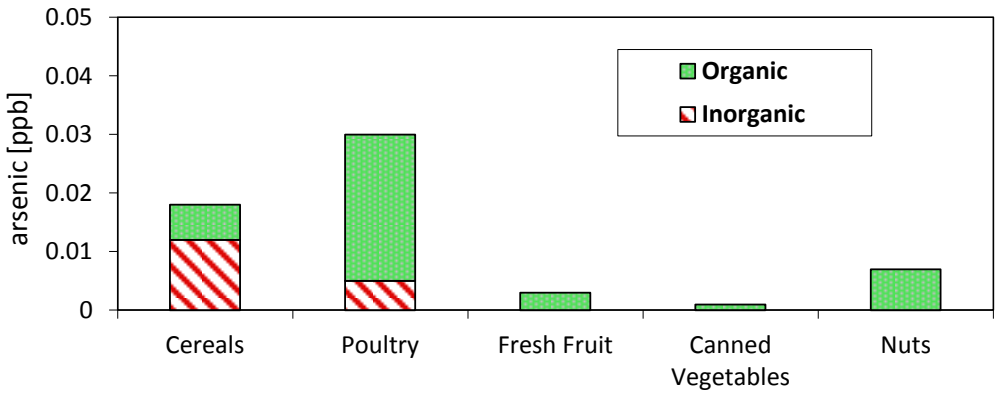


Figure 1-6: Proportion of inorganic and organic arsenic in food, derived from United Kingdom Food Standards Agency (FSA, 2009).

7.1 Arsenic in Rice

Plants rarely accumulate arsenic at concentrations harmful for human health because phyto-toxicity occurs before maturity. However, amongst cereals, rice is able to accumulate both inorganic and organic arsenic (Williams *et al.*, 2005a) when irrigated with water naturally enriched in arsenic (Williams *et al.*, 2006) or grown on soil contaminated with arsenic from anthropogenic sources (Abedin *et al.*, 2002b).

Rice that reaches the table of consumers in Europe and in the UK is grown in paddy fields of several parts of the world (see Figure 1-7). Under anaerobic conditions, occurring when the paddy fields are flooded with water, arsenic is mobilised as As(III) from soil to water and incorporated into rice plants (Takahashi *et al.*, 2004).

Total arsenic concentration in rice can range from 30 to 2050 µg/kg (Islam *et al.*, 2004b, Abedin *et al.*, 2002b, Abedin *et al.*, 2002a, D'Amato *et al.*, 2004, Mondal and Polya, 2008). Some total arsenic concentration values for raw rice are shown in Table 1-5 that has been modified from Williams *et al.* (2005a).

Table 1-5: Arsenic in rice sold in different countries of the world

Country	Source of Rice*	Total As in rice µg/kg (dw)	Reference
India (West Bengal)	G and P	130	(Mondal and Polya, 2008)
Bangladesh	G	143 (2-557)	(Rahman *et al.*, 2009).
Bangladesh		130(30-300)	(Williams *et al.*, 2005a)
China (polluted site)	G	490 (310-700)	(Xie and Huang, 1998)
Taiwan	G	200 (190-220)	(Schoof *et al.*, 1998)
USA		240(110-340)	(Heitkemper *et al.*, 2001)
USA		260	(Williams *et al.*, 2005a)
Europe		180(130-220)	(Williams *et al.*, 2005a)
Italy	G	(80-289)	(D'Ilio *et al.*, 2002)
Italy		220±10	(Williams *et al.*, 2005a)
Spain	G and P	114 ± 46	(Torres-Escribano *et al.*, 2008)
Spain		170 ± 10	(Williams *et al.*, 2005a)
Hungary	G	171.3 ± 7.1 139.0 ± 6.1 116.0 ± 3.7	(Mihucz *et al.*, 2007)

*G = grown; P = purchased; values in brackets represent the minimum and maximum reported, the first figure the average.

Rice contains both iAs (as AsIII and AsV) and organic forms (mainly DMA and MA) (Mandal and Suzuki, 2002, Williams *et al.*, 2005a) with variations within the organic/inorganic ratio from part of the world. It was recently discovered that rice contains tetramethylarsonium (Hansen *et al.*, 2011). One study even reported the presence of AB (Mandal *et al.*, 2007).

Arsenic is taken up as As(III) through the silicic acid transport pathway in rice (Ma *et al.*, 2008). Recently, researchers focused on understanding the uptake and sequestration of

Figure 1-7: Rice grown in the paddy fields is manually picked up and harvested. The de-husking process leads to polished rice that is cooked and eaten mainly in Asia and the rest of the world. Pictures from Bangladesh by P. I. Haris. This image was chosen for the cover page of Journal of Environmental Monitoring (vol. 31 No. 2 Feb 2011).

arsenic to develop strategies to reduce As concentration in the grain. Transporters belonging to the Nodulin-26-like intrinsic proteins (NIP) subfamily of aquaporins in rice

are permeable to arsenite AsIII but not to AsV. Mutations are induced in transporters to significantly decrease arsenite uptake (Ma *et al.*, 2008). A recent and elegant study from (Moore *et al.*, 2011) used High Resolution Secondary Ion Mass Spectrometry (NanoSIMS) and transmission electron microscopy (TEM) to study cellular and subcellular distributions of As and Si in rice roots. This study revealed the vacuolar sequestration of As in rice roots and contrasting patterns of As and Si subcellular localization, despite both being transported across the plasma membranes by the same transporters.

A geographical variation in the content of total and inorganic arsenic in polished white rice has been reported (Meharg *et al.*, 2009a). The total arsenic shows a 7-fold difference in median with rice from Egypt (0.04 mg/kg) and India (0.07 mg/kg) at the lowest limit and the one from U.S. (0.25 mg/kg) and France (0.28 mg/kg) with the highest levels. The relationship between inorganic arsenic *versus* total arsenic content varies significantly amongst different countries of origin, as shown in Figure 1-8. On a linear regression, Bangladeshi and Indian rice samples show the steepest slope (Figure 1-8) whilst rice from the U.S. has the shallowest slope. Inorganic species predominate in European, Bangladeshi and Indian rice, but not in American rice, where DMA(V) is the main species (Williams *et al.*, 2005a). This difference is likely related to the fact that inorganic As found in Asian rice has a geogenic origin while organic As in the American rice is due to the large scale use of As based pesticides at the beginning of 20th century.

Once rice is collected from the paddy fields, factors playing a role in the content of total arsenic in rice in the final meal include the de-husking procedure (Signes *et al.*, 2008b), cooking methods (Signes *et al.*, 2008a), rice variety and the type (Mondal and Polya, 2008) and quantity (Raab *et al.*, 2009) of water used for cooking.

Rice is a staple food for about half of the world's population and in some cases provides the main dietary calorific intake as for the population living in West Bengal and Bangladesh. Consumption of rice *pro capite* for Bangladeshis in their country of origin is 445 g /day (d.w.) (Meharg *et al.*, 2009c), while the rice consumption decreases to 251 g/day of rice (d.w.) when they move to the UK (Meharg, 2007). This intake is much higher than the intake for Europeans. The daily intake of arsenic from parboiled rice has been estimated to be 56.4 µg/day in Bangladesh (Rahman *et al.*, 2009).

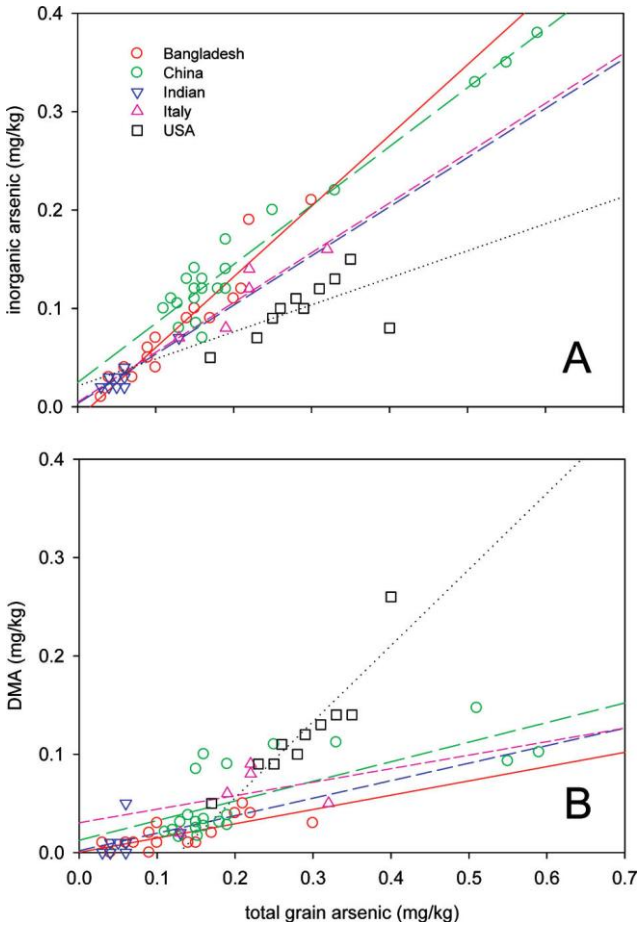


Figure 1-8: Regression analysis of inorganic arsenic and DMA against total arsenic concentrations in rice for countries from different regions of the world (Meharg *et al.*, 2009a).

Rice based products such as miso and amasake (Signes-Pastor *et al.*, 2009), rice milk, mixed cereals and baby food have been also investigated in an EU context (Sun *et al.*, 2009, Meharg *et al.*, 2008), revealing arsenic concentrations exceeding maximum content levels (MCL) set for drinking water by the EU regulation.

The EFSA in 2009 expressed its opinion on arsenic in food, highlighting the fact that some sensitive groups (including children, celiacs and ethnic groups) living in Europe are estimated to be exposed to an higher arsenic daily intake through the diet than the general population (EFSA, 2009).

Some ethnic groups who migrated to Europe still follow a traditional diet, based on rice and other foods imported from their country of origin, are estimated to be exposed to higher levels of arsenic (Al Rmalli *et al.*, 2005) than the general population. For instance,

the Bangladeshi community largely consumes traditional foods imported from Bangladesh. Al Rmalli *et al.* (2005) found a total As content of 54.5 µg/kg (range 5–540 µg/kg) in a survey of arsenic in foodstuffs on sale in the United Kingdom and imported from Bangladesh. Al Rmalli *et al.* conclude that As content of the vegetables imported from Bangladesh is 2- to 3-times higher than those from the UK. In this context, they suggested that it is likely the Bangladeshi community living in UK has a higher As daily intake than the general UK consumer, which is set at 61-64 µg/day by the Food Standards Agency (COT, 2008). Since Al Rmalli et al. (2005) did not provide any human bio monitoring study, the current study specifically investigated the effect of rice consumption on urinary arsenicals for a Bangladeshi community living in the UK compared to a group of White British Caucasians (see Chapter 3).

8. Anthropogenic arsenic

Anthropogenic As derives from industrial activities including mining, smelting of non-ferrous metals, burning of fossil fuels and the use of arsenic-containing pesticides (WHO, 2001). Organic arsenicals have been used as pesticides has and have led to environmental contamination. As compounds such as MMA(V), as sodium or calcium salt, and DMA(V), as sodium salt or free acid are herbicides widely used for weed control on cotton. The conversion or the rotation of cotton fields into rice fields carried out in US is the possible reason for arsenic contamination in American rice (Meharg *et al.*, 2009c).

Nowadays arsenic is used as a component of wood preservatives mainly as chromated copper arsenate (CCA)[1].

Arsenic Trioxide (As_2O_3) is used as a medication in the treatment of Acute Promyelocytic Leukemia (APL) via endovenous administration (Shen *et al.*, 1997).

9. Arsenic in the human body

Once inorganic arsenic is absorbed from the gastrointestinal tract, it enters the blood stream, and it is distributed to different organ/tissue compartments where it is involved in biochemical reactions causing change of molecular species, or it interacts with functional and structural molecules according to its chemical affinity. Once As(V) is in the blood it is

[1] (http://www.epa.gov/oppad001/reregistration/cca/).

rapidly reduced to As III (bio-activation), which is able to diffuse across the cell membrane because it is not dissociated at physiological pH (As(V) pKa1=2.2 and As III pka1=9.3) (Lerman *et al.*, 1983). Active transport mechanisms are known for As(III) (aquaglycoporins) and As(V) (phosphate transports) (Rossman, 2003). Arsenite As(III) shows higher toxicity than arsenate (AsV).

Post-mortem analysis of human tissues show that long-term arsenic exposure results in highest concentrations in the skin and lungs (0.01–1 mg/kg dry wt), as well as hair and nails (Liebscher and Smith, 1968; Cross *et al.*, 1979). In subjects exposed to high concentrations (0.2–2 mg/L) of arsenic in drinking-water, the concentration in liver is 0.6–6 mg/kg dry wt compared with 0.16 mg/kg for unexposed people (Guha Mazumder *et al.*, 1988). In the case of acute intoxication by arsenic, the liver and kidneys showed the highest concentrations of total arsenic with values 350- and 63-fold higher than those in blood, respectively (Benramdane *et al.*, 1999). In all organs, As(III) was the predominant species, and methylarsonic acid (MA (V)) occurred at higher concentrations than DMA (V). MA (V) and DMA were more prevalent in lipid-rich organs (49% and 45% of total arsenic in cerebellum and in brain, respectively) compared with other organs (20% of total arsenic). As (V) was found in small quantities in the liver, kidneys and blood (2% of total arsenic) (Benramdane *et al.*, 1999).

In humans, biomethylation of arsenic occurs mainly in the liver (Dopp *et al.*, 2005) and the enzyme arsenic(+III) methyltransferase (AS3MT) has been isolated from cytosol in hepatocytes (Lin *et al.*, 2002). Fibroblasts and urothelial cells do not express AS3MT, so As is mainly accumulated as iAs in these cells. Cyto and genotoxicity of Arsenic in hepatocytes is confirmed by the accumulation in nuclei and mitochondria (Dopp *et al.*, 2008). The main methylation sites are the liver, kidney, testes and lungs.

Different hypotheses have been postulated about As metabolism in humans, but despite the large number of studies carried out, there is still no universally approved mechanism for the metabolic pathways of arsenic *in vivo*.

According to Cullen's (Cullen and Reimer, 1989) model represented in Figure 1-9, As(III) is methylated to monomethylarsinousus acid (MA(V)) and sequentially, passing by reduction to monomethylarsenic acid (MA(III)), to dimethylarsinousus acid (DMAV). This double oxidative methylation reaction is catalyzed by arsenic(+III) methyltransferase (coded by the gene AS3MT) (Aposhian, 1997) (which corresponds to Cyt19 in the Human

Proteome Organization's (HUPO) classification) that uses S-adenosyl-L-transferase (SAM) as a methyl donor. A reductase, the glutathione S-transferase (GST O), is responsible for the reduction steps. Summing up, in this model first and second methylation steps are considered, and some authors report primary methylation index (PMI) as the ratio MAV/(As(III)+AsV) in urine and a secondary methylation index (SMI) as the ratio DMA(V)/ MA(V) in urine (Engström *et al.*, 2007). The first methylation step is considered to be the limiting methylation step in humans. Some studies correlate urinary arsenic methylation profile to the activity of the enzyme arsenic(+III) methyltransferase, levels of expression and SNPs of AS3MT (Engstrom *et al.*, 2011) .

The biomethylation step has been believed for years to be a biodetoxification mechanism for arsenic, considering the fact the As(III) was the most toxic species. However, Yamamoto *et al.* provided evidence for the fact that arsenic methylation is involved in a toxification process and produce intermediates like MA(III) and DMA (III) with highly toxic properties (Yamamoto *et al.*, 1995).

Based on the experimental evidence that Cyt19 (other name for the arsenic(+III) methyltransferase) produces more DMA (V) using As(III) as a substrate than from MA(III), Hayakawa *et al*. proposed an alternative pathway (Hayakawa *et al.*, 2005). In this second pathway (See Figure 1-9.B) arsine triglutathione (ATG) can be generated non-enzymatically from arsine As(III) and glutathione. ATG then becomes a substrate for the enzyme As(+III) methyltransferase that uses both SAM and GHS and that generate methyl arsine triglutathione (MADG). This reaction will either first give MA(III) and finally MA(V), or become a substrate for a second methylation reaction catalyzed by the As3MT leading to the production of DMAG. Hayakawa's model is compatible with the idea that methylation is a detoxification mechanism because the final products are just pentavalent methylated species.

Arsenic fatty acids have been found in urine after ingestion of cod liver (Schmeisser *et al.*, 2006). However, the analysis of arsenolipids in urine is not very common, especially due to the lack of suitable standards. Recently Raber *et al.* (2009) identified a protocol that uses gas chromatography Mass Spectrometry to identify arsenolipids in fish oil. The application of such kinds of approach could allow a greater characterization of food and biological samples for arsenolipids and help in risk assessment strategies.

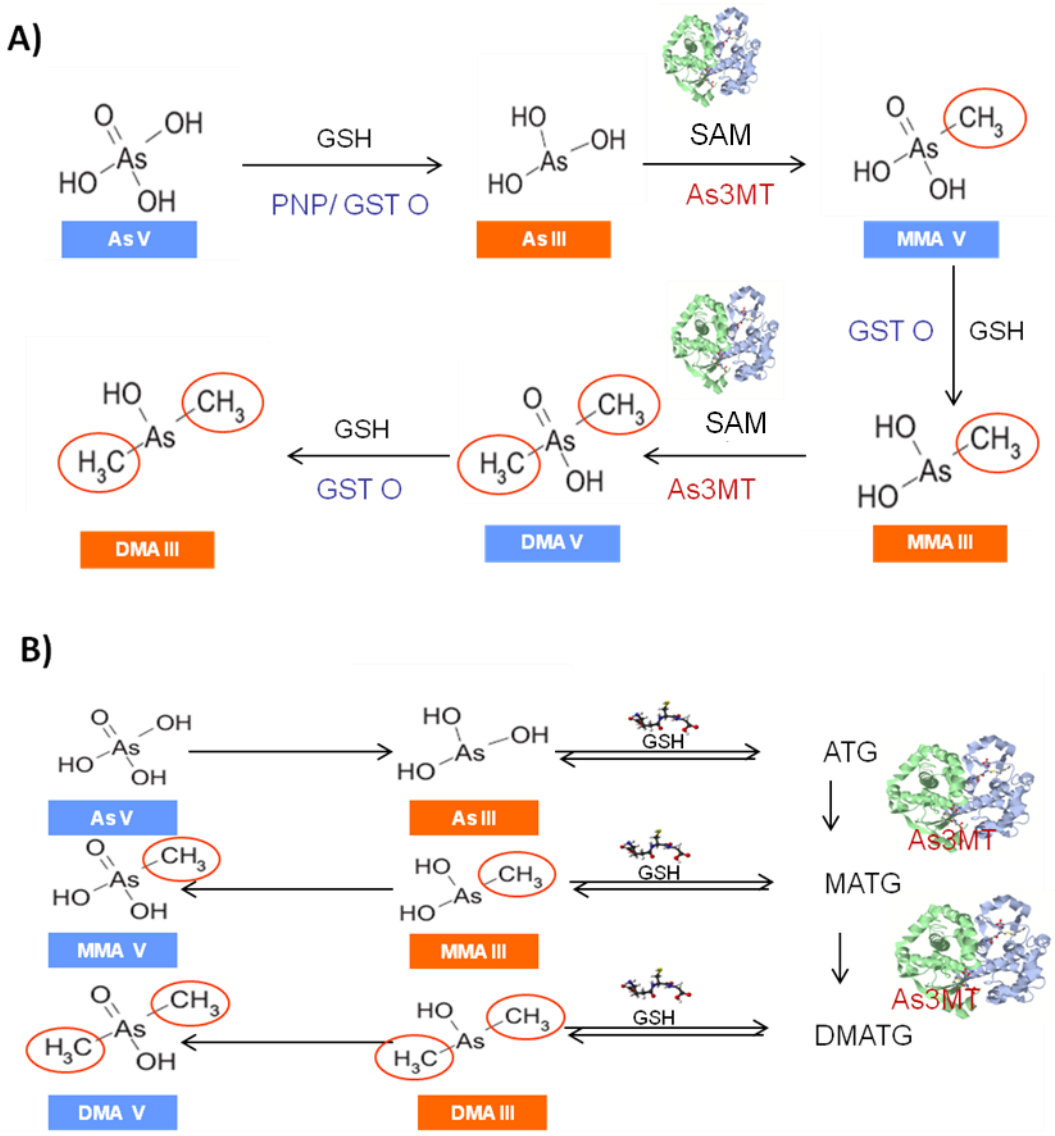


Figure1-9: Proposed models for bio-methylation of arsenic in humans; A) Traditional model as reported by Cullen (1989), Suzuki (2002), and Ghosh (2009). B) Alternative model as reported by Hayakawa, 2005 of arsenic methylation. The genes involved are depicted in red and the 3D structures of proteins are shown. Abbreviations: As3MT: gene coding for (Arsenic(+III) methyltransferase); SAM: S-adenosyl-L-transferase; GSH: glutathione; ATG: arsine triglutathione; MADG: methyl arsine triglutathione.

10. Health effects of arsenic exposure

An oral consumption of 70-180 mg of As_2O_3 in humans generally leads to death in 1 hour if not treated (Jones, 2007b). With regard to chronic effects, arsenic in drinking water is a carcinogenic agent for skin, lungs and bladder (IARC, 2004). Arsenic in drinking water may result in an increase in liver cancer mortality (Liaw *et al.*, 2008) (Liu and Waalkes, 2008).

Arsenic is also responsible for non-carcinogenic effects in humans (Calderon *et al.*, 2001) as detailed in Table 1-6.

Table 1-6: Carcinogenic and non-carcinogenic effects of arsenic exposure

Effect	**Type of study**	**Reference**
Skin, lung, bladder cancer	Review of current literature	(IARC, 2004)
Liver cancer	Review of current literature	(Liu and Waalkes, 2008)
Keratosis and Hyperpigmentation	Cross-sectional study	(Karim, 2000)
Neurobehavioral effects in e adolescents	Epidemiological	(Tsai et al., 2003)
Effects on memory and intellectual functions	Epidemiological	(Calderon *et al.*, 2001)
Increased foetal loss and premature delivery	Epidemiological	(Chakraborti *et al.*, 2003)
Steatosis	*In vivo* study, mouse	(Chen et al., 2004)
Cardiovascular diseases	*In vivo* study, rat	(Lee et al., 2002)
Ischemic heart diseases	Epidemiological	(Tseng *et al.*, 2003a)
Erythrocyte death	Epidemiological	(Biswas *et al.*, 2008)
Hypertension	Cross-sectional study	(Zierold *et al.*, 2004)
Respiratory system effects		(Milton and Rahman, 2002)
Depression	Cross-sectional study	(Zierold *et al.*, 2004)
Diabetes	Epidemiological	(Milton and Rahman, 2002)Tseng, 2002)

Smokers exposed to 200 µg/l of As(V) in drinking water have an increased risk of bladder cancer in comparison with smokers not exposed to such levels of As in drinking water,

suggesting a synergistic effect of the two factors in the cancer induction (Steinmaus *et al.*, 2006).

Furthermore, chronic exposure to arsenic in drinking water has been proved to be related to skin lesions such as keratosis and hyperpigmentation (see Figure 1-10). Skin lesions typically appear after 5-10 years and may develop into carcinogenic forms of the skin (non melanoma skin cancer) and of the internal organs (Yu *et al.*, 2007).

Keratosis and hyperpigmentation have been reported for some populations exposed via drinking-water, including West Bengal and Bangladesh (Karim, 2000), but not for other exposed groups such as those in Vietnam in the Red River Delta (Agusa *et al.*, 2009), in the Pannonian basin (Lindberg *et al.*, 2007b) or in the Andean population (Engström *et al.*, 2007). The Andean population of San Antonio de los Combres, exposed for thousands of years to As in drinking water (approximately 200 ppb), does not show any hyperkeratosis but shows an increase in urinary %DMA (Engström *et al.*, 2007). The reason for such variation in effects is thought to be related to: (i) duration of exposure (ii) ethnic differences (iii) genetic polymorphisms and different methylation (detoxification) ability.

Figure 1-10: Signs of arsenicosis (Meharg and Raab, 2010)

Along with the carcinogenic properties of arsenic, a number of non-carcinogenic effects have been proposed. Arsenic exposure may result in neurobehavioral and neuropathic effects in adolescence (Tsai et al., 2003), effects on memory and intellectual function

(Calderon et al., 2001), reproductive effects with increased foetal loss and premature delivery (Chakraborti *et al.*, 2003) (Milton *et al.*, 2005), steatosis (Chen *et al.*, 2004), cardiovascular diseases (Lee *et al.*, 2002), ischemic heart diseases (Tseng *et al.*, 2003b), carotid atherosclerosis and respiratory system effects such as chronic cough and chronic bronchitis (Milton and Rahman, 2002). Even at concentrations as low as 0.4 μg/L, As(III) has been reported to behave as an endocrine disruptor that is able to alter gene transcription (Bodwell *et al.*, 2004). Despite the number of *in vivo* and *in vitro* studies trying to elucidate the role of As in the development of diabetes in humans (Tseng *et al.*, 2002), the current available evidences are not adequate to establish a causal role (Chen *et al.*, 2007a). After adjustment for biomarkers of seafood intake, total urine arsenic was found associated with increased prevalence of type 2 diabetes by (Navas-Acien *et al.*, 2008). The authors (Navas-Acien *et al.*, 2008) suggest that their findings support the hypothesis that low levels of exposure to inorganic arsenic in drinking water, a widespread exposure worldwide, may play a role in diabetes prevalence. However, more recent studies suggest that there is no link between arsenic exposure and diabetes (Argos *et al.*, 2010, Chen *et al.*, 2010).

Consequences of chronic exposure to arsenic via drinking water in humans depend on dose, duration and species (Tchounwou *et al.*, 2004). Other factors playing a role, to differing extents, in reported effects are related to the individual's susceptibility like ethnicity and diet. The large variety of effects reported in different geographic areas and sub-population groups, apparently exposed to the similar levels of As in drinking water, indicate that there is a level of uncertainty in this matter. Two main, uncertain factors can play a role, and are actually considered in the formulation of the risk assessment for As in drinking water: (i) variability that is related to inter-individual differences and susceptibility; (ii) uncertainty related to which data are currently used for the estimation of the dose-response relationship.

11. Arsenic limits and intakes

In 1963, WHO set a first maximum content level in drinking water at 50 ppb, on the basis of its proven toxicological properties and carcinogenicity. This value was lowered to 10 ppb in 1992 on the basis of further findings. Consequently, several countries decreased As MCL in drinking waters from 50 to 10 ppb including USA (since 2006), EU (since 2003), Canada and Taiwan (since 2000). For various reasons, mainly due to the cost of

technology required for reducing As levels in drinking water, other countries such as Bangladesh, India and China have maintained the MCL at 50 ppb. In some of the states of the USA, it has not been possible to fully implement the 10 ppb level[2].

A no-observed-adverse-effect level (NOEAL)[3] for inorganic arsenic was derived by the United States Environmental Protection Agency (USEPA) from a number of studies on drinking-water. USEPA mainly considered the studies by Tseng (Tseng, 1977, Tseng *et al.*, 1968b) because of the large study population and the dose-related incidence of skin lesions. The NOAEL, derived from an estimation of the exposure of the portion of the study population that did not develop skin lesions, was set at 0.8 µg/kg b.w. per day. For inorganic arsenic, a PTWI of 15 µg/kg b.w. was established by JECFA (WHO, 1988). However, the CONTAM Panel (EFSA, 2009) has more recently evaluated this limit as no longer appropriate because data has shown that inorganic arsenic causes cancer of the lung and bladder in addition to skin, and a range of adverse effects at exposures lower than those originally reviewed by the JECFA. After reviewing key epidemiological studies, the CONTAM Panel modelled the dose-response data and selected a benchmark response of 1 % extra risk. A range of benchmark dose lower confidence limit ($BMDL_{01}$) values between 0.3 and 8 µg/kg b.w. per day were identified for cancers of the lung, skin and bladder, as well as skin lesions.

For organic arsenicals limited data are present in experimental animals; arsenobetaine (AB) is thought not to be of toxicological concern. For oral administration of methylarsonate, NOAEL (developmental toxicity based on pregnancy outcome) are 100 and 7 mg/kg b.w. per day in the rat and the rabbit, respectively (ATSDR, 2007). For oral dimethylarsinate reported NOAEL values are 12 mg/kg b.w. per day in the rat and the rabbit (ATSDR, 2007).

Currently, the vast majority of cancer slope factors for ingested inorganic arsenic used by the main agencies to calculate the 'safe values' for arsenic in drinking water rely on the idea that As follows a linear, non-threshold relationship. Epidemiological data available for populations exposed to high levels of arsenic (mainly Taiwan's) are used to extrapolate a

[2] http://pubs.usgs.gov/fs/2000/fs063-00/fs063-00.html#HDR1

[3] NOEAL is defined as the greatest concentration or amount of a substance, found by experiment or observation, which causes no detectable adverse alteration of morphology, functional capacity, growth, development, or life span of the target organism under defined conditions of exposure (http://goldbook.iupac.org/NT06911.html).

linear curve, from which a low level dose-effect response is estimated. Some authors have strongly criticised this approach (Boyce *et al.*, 2008), basing their criticism on the following: (i) As does not induce carcinogenicity through direct interaction with DNA but via indirect toxicity mechanism possibly relying on inhibition of DNA repair, induction of dysfunctional cell division, perturbation of DNA methylation patterns, modulation of signal modulation pathways (ii) As carcinogenic mode of action is not linear and therefore is not governed by additivity to a background model, (iii) some studies suggest the presence of a threshold level for carcinogenic effects. Clearly, further work needs to be carried out in this area to resolve the controversy.

12. Factors regulating bioavailability, uptake and effects of arsenic

It has been reported that populations exposed to similar levels of As in drinking water living in different countries may develop different pathological outcomes (see Table 1-6). The inter-individual variability and susceptibility to As toxicity is sometimes attributed to variations in individual methylation ability. Therefore the relative abundance of methylated As species in urine is used as a proxy indicator of individual As susceptibility. Chapter 3 will present results that can affect this postulate. The evaluation of inter-individual variability is one important component in risk assessment, as highlighted by US National Research Council (NRC, 2001) and includes the evaluation of differences based on life style, diet, smoking habits, water consumption rate and ethnicity. Some factors thought to have an effect on methylation pathways and pathological outcomes are shown in Figure 1-11.

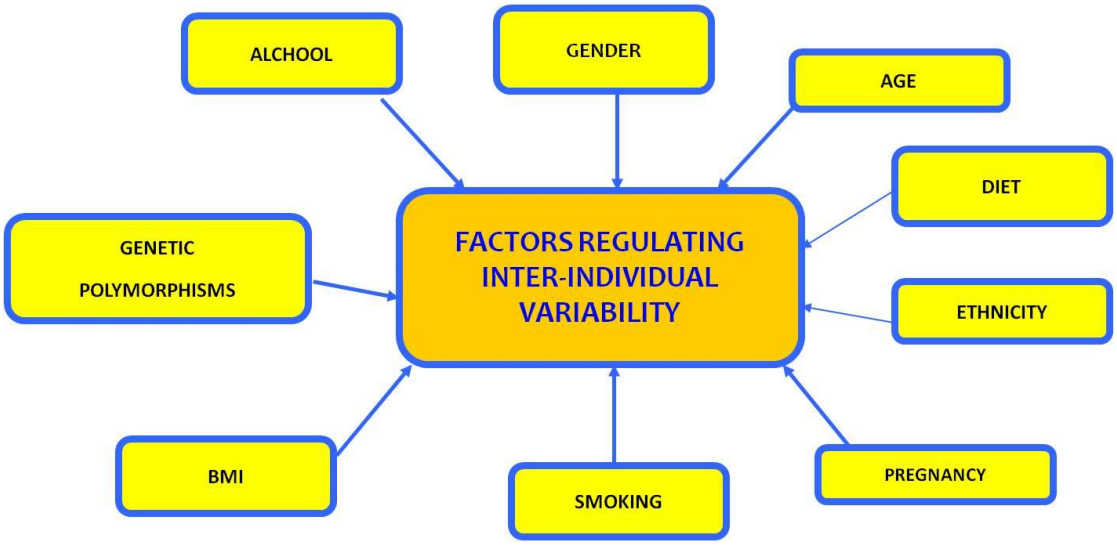


Figure 1-11: Factors having an impact on arsenic susceptibility/metabolism in humans, after (Tseng, 2009).

12.1 Diet and life style

Nutritional status may play an important role in the regulation of arsenic methylation and arsenic related health hazards in humans.

Pierce *et al.* report that skin lesion risk varies with different dietary patterns in affected populations of Bangladesh; eating a diet rich in gourds and root vegetables and increasing dietary diversity may reduce arsenical skin lesion risk in Bangladesh (Pierce *et al.*, 2011).

Chandra (Chandra *et al.*, 2003) reports a positive correlation between the consumption of low nutritious food and increased As toxicity. Undernourishment, such as a low intake of animal proteins, calcium and fibers have been found to be associated with skin lesions and cancer (Hsueh *et al.*, 1997, Mitra *et al.*, 2004). A study of a population in West Bengal (India) shows that the prevalence of skin lesions in people with the lowest intake of certain nutrients was approximately two times higher (Mitra *et al.*, 2004). Pilsner (Pilsner *et al.*, 2009) recognizes folate deficiency, hyperhomocysteinemia, low urinary creatinine and hypomethylation of leukocyte DNA as risk factors for arsenic induced skin lesions. Mitra (Mitra *et al.*, 2004) reports in a case-control study in West Bengal, that arsenic related skin lesions were more commonly seen in arsenic-exposed subjects with low dietary folate intake. Gamble *et al.* reported that folate could facilitate arsenic methylation and that folate supplementation could enhance arsenic methylation, decrease blood MA

and increase urinary DMA (Gamble *et al.*, 2007). A study in Taiwan confirmed that high plasma folate level was associated with a lower risk of urothelial cancer (Huang *et al.*, 2008).

Vitamin C and methionine reduces As toxicity and deficiency of vitamin A increases sensitivity to As (Roychowdhury *et al.*, 2002). Heck *et al.* (2007) indicates that there is no significant relationship between nutritional intakes and the percentage of DMA measured in urine, but significant association was observed between dietary intakes of methionine, vitamin B-12, calcium, protein, riboflavin, and vitamin A and the percentage of monomethylarsonous acid (MA) in urine.

Table 1-7: Dietary Factors related to As toxicity/metabolism (↑= increase; ↓=decrease) (after Tseng *et al.,* 2009)

FACTOR	RELATED WITH	EFFECT	REFERENCE
Intake of food with low nutrition value	As toxicity	↑	(Chandra *et al.,* 2003)
Low intake of animal proteins, Ca, fibers	Skin lesions and cancer	↑	(Hsueh *et al*., 1997, Mitra *et al.,* 2004)
Folate deficiency	Skin lesions	↑	(Pilsner *et al*., 2009)
Low Folate intake	Skin lesions	↑	(Mitra *et al*., 2004)
Folate supplementation	Arsenic methylation, decrease blood MA, increase urinary DMA	↑	(Gamble *et al*., 2007)
High plasma folate	Risk of urothelial cancer	↓	(Huang *et al*., 2008).
Vitamin A	Sensitivity to Arsenic	↑	(Roychowdhury *et al.,* 2002) (Chandra *et al.,* 2003)
Vitamin C and methionine	Resistance to As toxicity	↑	(Roychowdhury et al., 2002)
Intake of methionine, vitamin B-12, calcium, proteins, riboflavin, vitamin A	% of MA in urine	↑	(Heck *et al.,* 2007)
Blood Selenium	Incidence of skin lesions	↓	(Chen *et al.,* 2007b)
Rich diet, root vegetables and dietary diversity	Arsenical skin lesion risk	↓	(Pierce *et al.*, 2011)
Betel nut use	Risk of developing skin lesions	↑	(McCarty *et al.*, 2005).

Serum *β*-carotene level can modify the extent of risk association between methylated arsenic species and skin cancer (Hsueh *et al.*, 1997) and ischemic heart disease (Hsueh *et al.*, 1998).

Urinary creatinine is positively associated with DMA percentage in studies conducted in Bangladesh (Gamble *et al.*, 2005, Li *et al.*, 2008). Creatinine is used to normalize the urine according to their dilution, and a proxy indicator of nutritional status because it is associated with muscle mass and meat intake. Because creatine, which is a precursor of creatinine, is formed during arsenic methylation while using S-adenosylmethionine as a methyl donor, the use of creatinine to normalize urine and as a proxy of nutritional status requires better evaluation (Li *et al.*, 2008).

Finally, an inverse correlation between BMI and the risk of adverse health effects from arsenic exposure, is reported from (Islam *et al.*, 2004a). Body fat content may therefore have an effect on the storage of arsenic and therefore may exert an impact on the metabolism of arsenic.

It is important to note that from a European context, no visible symptoms (such as keratosis & hyperkeratosis) have been reported for populations that are known to be exposed to arsenic through drinking water (Romania, Hungary & Slovakia). It is possible that better nutrition and/or genetic factors may be responsible for absence of such symptoms since epidemiological studies suggest a possible increase in cancer rates in such populations.

12.2 Smoking and Chewing Betel quid[4]

Cigarettes contain arsenic and it has been estimated that smoking one cigarette will result in inhalation of about 0.25 μg of arsenic (ATSDR, 1993). Cigarette smokers have significantly higher urinary total arsenic, MA(V) percentage and than non-smokers (Tseng, 2005). This presumably indicates that smokers have a lower methylation capacity than non smokers. Smokeless tobacco, through chewing with or without betel nut can represent another source of exposure to arsenic and other elements (Al-Rmalli *et al.*, 2011b). Betel nut use appears to be associated with increased risk of developing skin lesions in Bangladesh (McCarty *et al.*, 2005).

[4] A combination of betel leaf, areca nut, and slaked lime.

12.3 Drinking alcohol, tea and coffee

Alcohol drinkers show a urinary arsenic profile indicating a poorer methylation capacity compared with non-drinkers (Hopenhayn-Rich *et al.*, 1996).

Some investigators consider tea and coffee consumption as potential confounders (Paiva *et al.*, 2008). However the effect of coffee on urinary arsenic profile seems to be irrelevant (Tseng, 2009). Tseng suggest that it is inconclusive whether or not these daily common beverages could exert an effect on arsenic metabolism and more research work is required to provide an answer.

12.4 Ethnicity, age and gender

As highlighted before, the usual proportion of arsenic metabolites in human urine is 10-30% inorganic As, 10-20% MA (V) and 60-80% DMA (V) (Brima *et al.*, 2006c). Variations to this "usual" proportion are reported in some populations. For instance, ethnic origin has been related to different urinary methylation patterns in different ethnic groups living in Leicester (UK) by Brima *et al*. (Brima *et al.*, 2006b), exposed to the same level of As via drinking water (As<10ppb). A significant difference ($P<0.01$) in the proportion of AB, DMA and total arsenic was found in black-Somali population compared to two control population groups (Asian and White). Further studies are required to understand if such differences are due to ethnicity or dietary habits. This issue has been addressed in Chapter 3 where the arsenic urinary profile of UK Bangladeshi and white Caucasians has been compared. The Andean population of San Antonio de los Combres, exposed for thousands of years to a high content of As in drinking water (about 200 ppb), do not show any hyperkeratosis but reveal an increase in urinary %DMA. These differences have been investigated with the study of genetic polymorphisms (see the next section). The authors (Engström *et al.*, 2007) hypothesize a positive selection of a genotype to be involved in methylation pattern modification and less toxicity manifestation

In the Red River Delta region of Vietnam, Agusa (2009) reports a higher concentration of DMA and total arsenic in urine of male children compared to adult males. Furthermore, a positive correlation with age and sex is reported. Such a trend is not seen in female children.

Children present a higher second methylation rate and a major excretion of DMA (Chowdhury *et al.*, 2003). In fact, comparing the urinary arsenic of adults and children

below 11 years of age, the latter seem to retain less arsenic in body tissues such as hair and nail, and excrete arsenic in urine more efficiently. Other studies show a higher concentration of MA in children compared to adults. The current studies therefore do not confirm a unique trend and do not allow a full picture of the relationship between age and methylation capacity (Tseng, 2009).

A few studies (Lindberg *et al.*, 2007b, Lindberg *et al.*, 2008, Steinmaus *et al.*, 2005b) suggest that women have a better methylation capacity than men. Those who are over 60 years and overweight/obese women exposed to low dose-inorganic As in drinking water had a greater methylation efficiency compared to males (Lindberg *et al.*, 2007b) suggesting the role of estrogens in methylation. A proposed metabolic pathway that link estrogens and dietary factors with As metabolism can be found in Figure 12 In this pathway, choline synthesis (which is up-regulated by estrogens) leads to the synthesis of betaine. This can donate its methyl group to homocysteine to form methionine, which is involved in As methylation pathway.

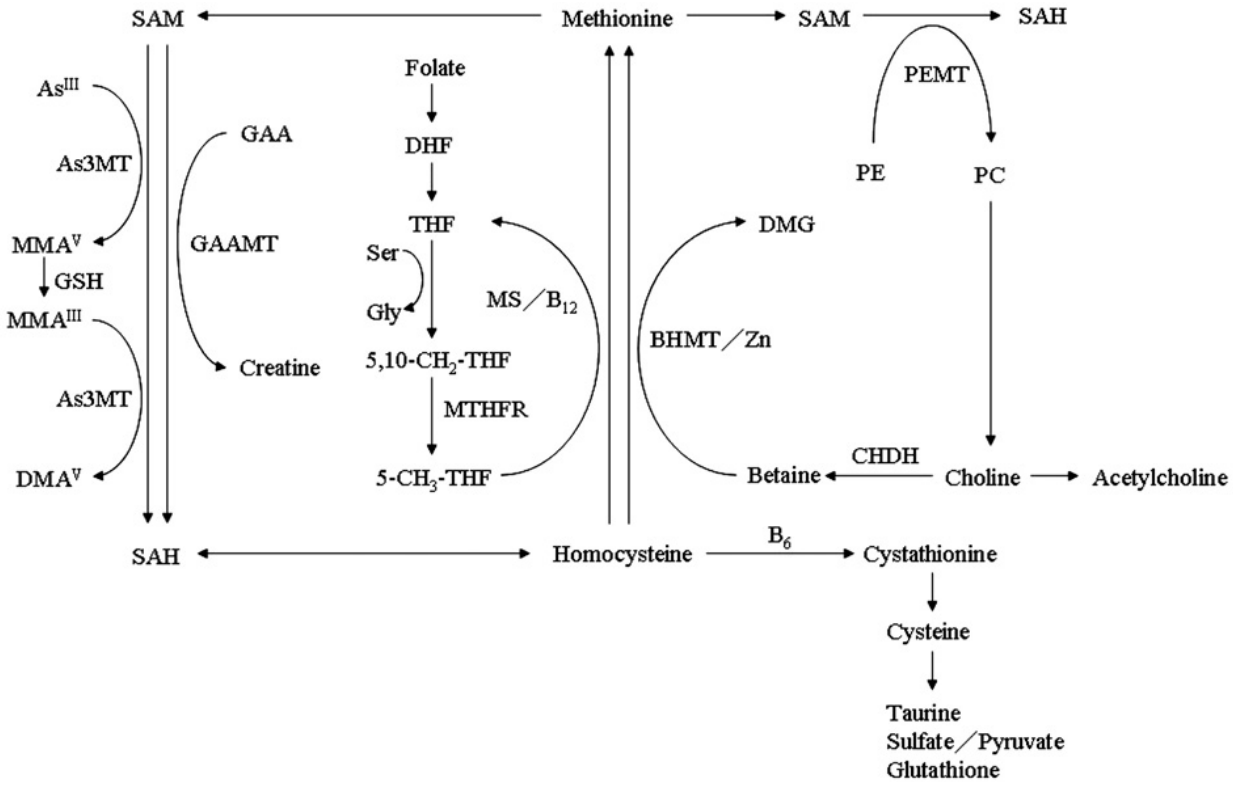


Figure1-12 : Metabolic pathways linking estrogen, folate, choline, betaine, B12 and arsenic methylation from (Tseng et al. , 2009); (PEM pathydilethanolamine methyltranferase; BHMT= betaine-homocysteine methyltranserase; CHDH= choline dehydrogenase; DMG = dimethylglycine; GAA= guanidinoacetate; GAAMT= guanidinoacetate methyltransferase; MS= methionine synthase; MTHFR= methylenetetrahydrofolate reductase; PC = phosphatidylcholine; PE = phosphatatidylethanolamine; PEMT = phosphatidylethanolamine-N-methyltransferase; SAH = S-adenosylhomocysteine; THF = tetrahydrofolate; 5,10-CH2-THF = methylene tetrahydrofolate; 5-CH3-THF = methyl tetrahydrofolate)

12.5 Genetic polymorphisms

Genetic polymorphisms represent variations in the coding region of DNA, that might result in the alteration of protein/enzyme activity. If this alteration affects one single nucleotide, it is defined as a single nucleotide polymorphism. Several genomics studies have tried to relate genetic polymorphisms with the capacity of arsenic methylation and health outcomes in affected populations.

A recent study demonstrated that polymorphisms in *AS3MT* significantly predicted As metabolism across two very different populations, one from South America and one in Southeast Asia (Engstrom *et al.*, 2011).

13. Toxicity and Essentiality of Trace elements

The theoretical dose-effect relationship in humans (EVM, 2003) is presented in Figure 1-13; for *essential* TEs such as selenium, a decrease in exposure below the acceptable range of intake leads to a deficit in biological functions resulting in deficiency (on the left side), with the slope of the curve proportional to the severity of the effects. On the other side an increase of exposure above the NOEAL leads to increasing *toxicity* as the exposure increases in accordance with the slope of the curve. For both essential and non essential trace elements, the lowest-observed-adverse effect level (LOAEL)[5] can be identified on the right-hand section of the curve.

As remarked by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA, October 2004), this graphic representation is helpful in making the point about the two-tailed potential for harm (also known as Giano effect), but the dose-response relation may not be symmetrical (as indicated by the dotted lines) and the shape of the curves may differ being highly element and sometimes species-specific.

[5] LOAEL it is generally defined as the lowest concentration or amount of a substance, found by experiment or observation, which causes an adverse alteration of morphology, functional capacity, growth, development, or life span of a target organism distinguishable from normal (control) organisms of the same species and strain under defined conditions of exposure (http://goldbook.iupac.org/LT06909.html).

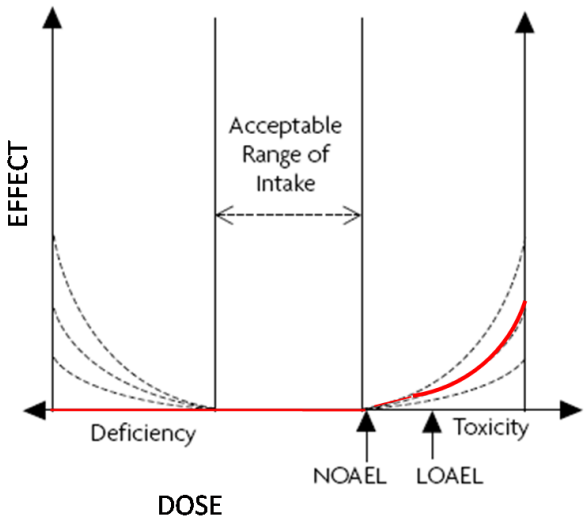


Figure 1-13: Dose-response curve for essential (dotted-black) and non essential (red) trace elements. NOAEL is the no-observed-adverse-effect level and LOAEL is the lowest-observed-adverse effect level (modified from (EVM, 2003).

The exposure to an *acceptable* level of intake represents the ideal condition. However, it is often impossible to reach this *optimum* and even for carcinogenic trace elements, such as As or Pb, it is unfeasible to have an environment absolutely contaminant free.

For essential and non essential trace elements different authorities use different parameters and methodologies for predicting the safe dose and the 'borders' between deficiency and toxicity. There is in general a lack of homogeneity across nations and regulatory bodies. Among the most used set parameters to define the maximum levels of contaminants 'allowed' for humans, JECFA[6] defines the following:

- ***"Acceptable Daily Intake* (ADI)** *is used for substances that can be relatively easily controlled in the food supply. The ADI is an estimation of the amount of a substance in food or drinking water, expressed on a body-weight basis (mg per kg of body weight, standard human = 60 kg), that can be ingested daily over a lifetime without appreciable risk for health.*
- ***Provisional Tolerable Weekly Intake* (PTWI)** *is used for food contaminants such as heavy metals with cumulative properties in the body; the weekly designation stresses the importance of limiting the dose over a period of time. PTWI represents*

[6]Quotations from http://www.who.int/ipcs/food/jecfa/glossary/en/

permissible human weekly exposure to those contaminants unavoidably associated with the consumption of otherwise wholesome and nutritious foods. The term 'tolerable' stresses the permissibility rather than the acceptability. Moreover, the term provisional underlines the tentative nature of this evaluation.

- ***Provisional Maximum Tolerable Daily Intake (PMTDI)*** *is established for essential trace elements with no cumulative properties. Its value represents permissible human exposure as a result of the natural occurrence of the substance in food and in drinking-water. In the case of trace elements that are both essential nutrients and unavoidable constituents of food, a range is expressed, the lower value representing the level of essentiality and the upper value the PMTDI"*

Some examples of PTWIs and PMTDIs are reported in Table 1-8.

Table 1-8: PTWIs and PMTDIs for some essential and non essential trace elements

Element	PTWI	PMTDI	Reference
Aluminium	7 mg/kg bw		(JECFA, 1989)
Arsenic	*0.015 mg/kg bw		(WHO, 1988)
Copper		0.5 mg/kg bw	(JECFA, 1982a) (JECFA, 1982b)
Zinc		1 mg/kg bw	(JECFA, 1982a)

*This limit is no longer considered appropriate (EFSA, 2009).

14. Uptake, bioaccessibility and bioavaialability

Routes of human exposure to trace elements include dermal exposure, ingestion and inhalation. Ingestion of naturally contaminated groundwater exploited for drinking purposes (Berg *et al.*, 2001), inhalation of volcanic gas emissions and dusts (Ng'walali *et al.*, 1999) and accidental soil ingestion (Ljung *et al.*, 2006) are just few examples of exposure routes.

The study of the uptake of trace elements in humans requires knowledge of two basic concepts: bioaccessibility and bioavailability (see Figure 1-14). In general, *oral bioaccessibility* represents the fraction of the contaminant that is soluble in the gastrointestinal tract and available for absorption, while *oral bioavailability* is defined as the fraction of the contaminant that reaches the systemic circulation from the gastrointestinal tract (Girouard and Zagury, 2009). There are four steps involved: absorption, distribution, metabolism and excretion. The application of a toxicokinetic

approach helps in the understanding of the behaviour of trace elements once they enter the organism.

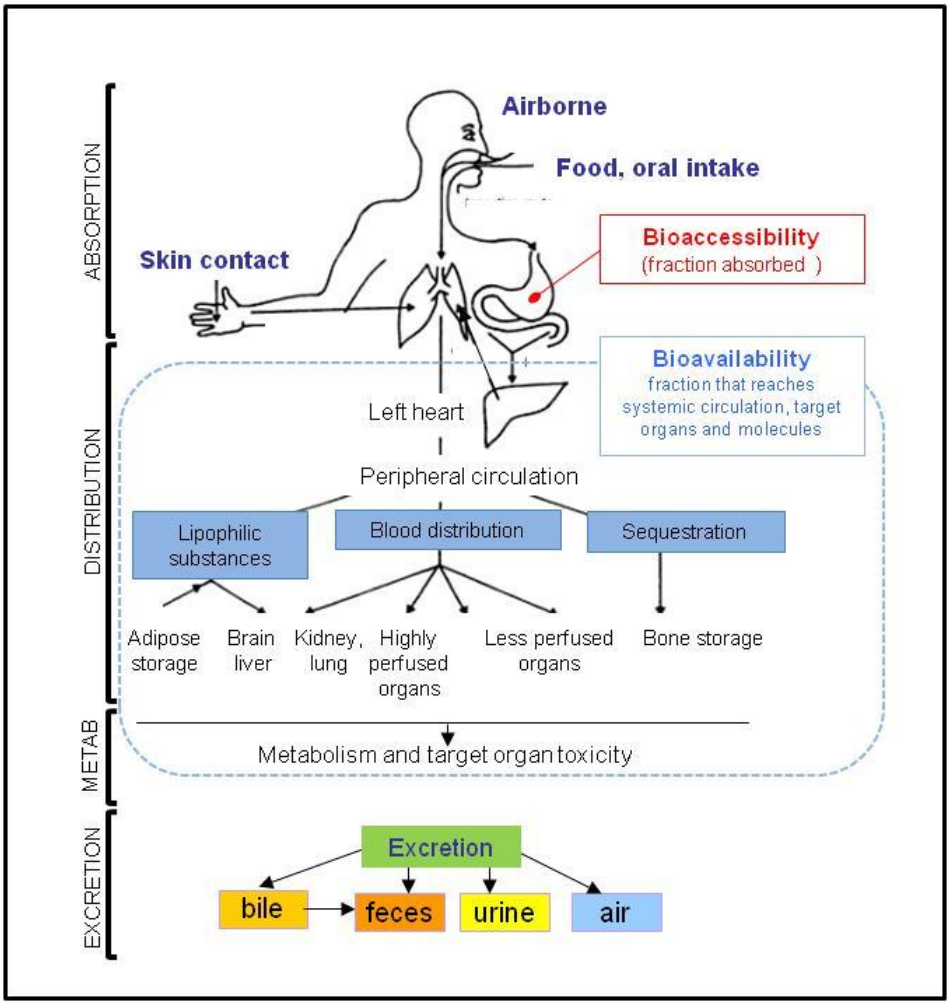


Figure 1-14: Toxicokinetics, bioaccessibility and bioavailability in humans (modified from Guidotti, 1994)

Both the stomach and the intestine are responsible for absorption of trace elements in the gastrointestinal tract involving several biological processes, i.e. active or passive transport, use of receptors from some trace elements that regulate the quantity absorbed. The change of environmental conditions (pH, Eh, oxygen content, etc) from one compartment to the other compromises the speciation and fate of a date trace element.

Factors affecting gastrointestinal absorption of trace elements in humans can be classified into: i) *extrinsic or dietary* (related to the elements to be absorbed) and ii) *endogenous or physiological* (related to the organism that absorbs) (WHO, 1996) (see Table 1-9).

Extrinsic or dietary factors include: pKa of the ingested compound, valence state, the presence of antagonists and synergists and methylation state. In general, neutral compounds are more readily absorbed than the ionized ones. With regards to extrinsic factors, the solubility and the molecular dimension is important in regulating what can pass through the GI tract (and how). Moreover, some substances (known as synergists) enhance absorption whilst others decrease it, limiting the mobility and the bioavailability.

Table 1-9: Factors involved in human uptake of trace elements, after WHO, 2006

Extrinsic factors	
Enhancing absorption (e.g. citrate, histidine enhance zinc) **Maintaining systemic transport** (maintaining systemic transport)	Antagonists Decreasing GI lumen solubility Competing with the element for receptors of adsorption Synergists
Endogenous factors	
Absorption process	**Metabolic/functional interaction**
Development changes (i.e. infancy *vs* senility) Homoeostatic regulation For example, response to high demand during pregnancy though modification of activity/concentration of GI receptors)	• Inter-dependence of elements on body mobility/storage • Metabolic processes reducing mobility • Processes enhancing release

In the endogenous group, physiological processes (related to both absorption and metabolism) that influence bioavailability, utilization and effects are included. Changes in absorption can be related to age, for example zinc absorption in the age range 22-30 is 33% opposed to 17% between 65 and 77; or to particular conditions like pregnancy when a modification of the abundance/activity of receptors in the GI tract leads to better absorption of some nutrients such as Cu (WHO, 1996). Mechanisms of absorption rely on diffusion through the membrane (for lipophilic substances), or specialized transport; facilitated diffusion of divalent metal-ion transporters (such as DMT-1) is known for several divalent cations including Cd, Co, Cu, Fe, Mn, Pb, and Zn in the intestinal tract, regulating intestinal uptake (Gunshin *et al.*, 1997). Transferrin and its interaction with the

transferrin receptor plays a role in the movement of Fe across membranes (Hentze *et al.*, 2004). The multidrug resistance-associated protein-2 (MRP2) is thought to be involved in biliary transport of glutathione complexes of As. Selenomethionine is transported in the intestine by the same transporter that mediates methionine uptake (Moesgaard and Morrill, 2001).

Homeostatic mechanisms are associated with nutrient substances. They operate at both low and high levels of intake to maintain the amount of nutrient substance in the body within an acceptable range.

Metabolic interactions influencing bioavailability can arise from involvement in metabolic pathways of other trace elements (for example, the interaction between selenium and iodine). This can result in a reduction or enhancement of mobility and/or release from a storage form to a bioavailable form. A widely studied interaction is the one between arsenic and selenium observed both in animal studies (Zeng *et al.*, 2005), and humans (Zeng *et al.*, 2005). Several mechanisms have been proposed but the actual process remains unclear.

15. Other trace elements

A brief overview of selected trace elements and their implications for human health follows, focusing on geogenic origin, biochemical and physiological processes related to essentiality and the main effects on humans arising both from deficiency and from overexposure. This overview is also summarised in Table 1-10.

15.1 Selenium

Selenium (Se) essentiality is due to its involvement in the structure of selenium cysteine, which is present in several human enzymes such as glutathione peroxidases, iodothyronine deiodinases, tiredoxixin reductase, selenophosphate synthase-2.

Selenium intake varies depending on the selenium content in the soil. A deficit in Se intake caused by food grown in selenium-depleted soils, results in adverse effects on the human body such Keshan disease, an endemic cardiomyopathy affecting children in an area of China (Yang et al.1998). Conversely, an overexposure to selenium results in loss of hair and fingernails (Yang et al., 1983) and nervous system abnormalities.

The lower limit of safe range for selenium has been fixed at 40 μg/day and the upper at 400 ug/day by WHO, considering the essential/toxicity paradigm (WHO, 1996). Extreme values from below 10 μg/day up to 5 mg/day, both from China, have been reported (Combs Jr, 2001). In Western countries, such as Denmark, a European country, the daily intake is 38-47 μg, whereas on the North American continent, the daily intake in the USA is 106 μg/day and it is 98–224 μg/day in Canada (Rayman, 2008).

Se is an essential metalloid for the human body. It is a part of a range of enzymes including gluthathione peroxidase, which is involved in the antioxidant system of the body and thioredoxin reductase, which participates in the antioxidant protection of cells (Birringer *et al.*, 2002). In addition, some studies suggest that some organic forms of selenium could show anticarcinogenic properties against certain types of cancer (Shen *et al.*, 2006).

Se belongs to group 16th of the periodic table, along with O, S and Te. The geochemistry of Se is similar in most respects to that of S, although Se is a much less abundant element. Se compounds of interest in plants include inorganic species such as selenite Se(IV) and selenate Se(VI); simple organic species including methylselenol, dimethylselenide, diethylselenide and dimethyloselenooxide; amino acids and low molecular species such as selenomethionine (SeMet), selenocysteine (SeCys), selenocystine (SeCys2), selenohomocysteine (SeHoCys2), Se-methylselenocysteine (MeSeCys), Se-methylselenomethionine (MeSeMet), Se-allylselenocysteine (AllSeCys), Se-propylselenocysteine (PrSeCys), γ-glutamyl-Se-methylselenocysteine γ-Glu-MeSeCys), Se-adenosylselenohomocysteine, selenoglutatione; and other compounds such as selenoproteins or selenoenzymes. Some selenium species are shown in figure 1-15 A and B. Se-Methylseleno-N-acetylgalactosamine (SeGalNAc) (see Figure 1-16) is the major urinary metabolite in rat and human urine and is excreted after ingestion of selenite, SeMet and yeast (Gammelgaard *et al.*, 2011).

Se, along with Iodine (I), affects homeostasis of thyroid hormone-dependent metabolic pathways. Endocrine tissues such as thyroid, adrenals, pituitary, testes and ovary shows higher selenium than in many other organs. Several selenocysteine-containing proteins participate in the protection of thyroid cells from damage by H_2O_2 produced for thyroid hormone biosynthesis. Iodothyronine deiodinases are selenoproteins contributing to systemic or local thyroid hormone homeostasis. Hormones and growth factors regulate the expression of selenoproteins and, conversely, Se supply modulates hormone actions.

Spermatogenesis depends on an adequate Se supply, whereas Se excess may impair ovarian function (Koehrle *et al.*, 2005).

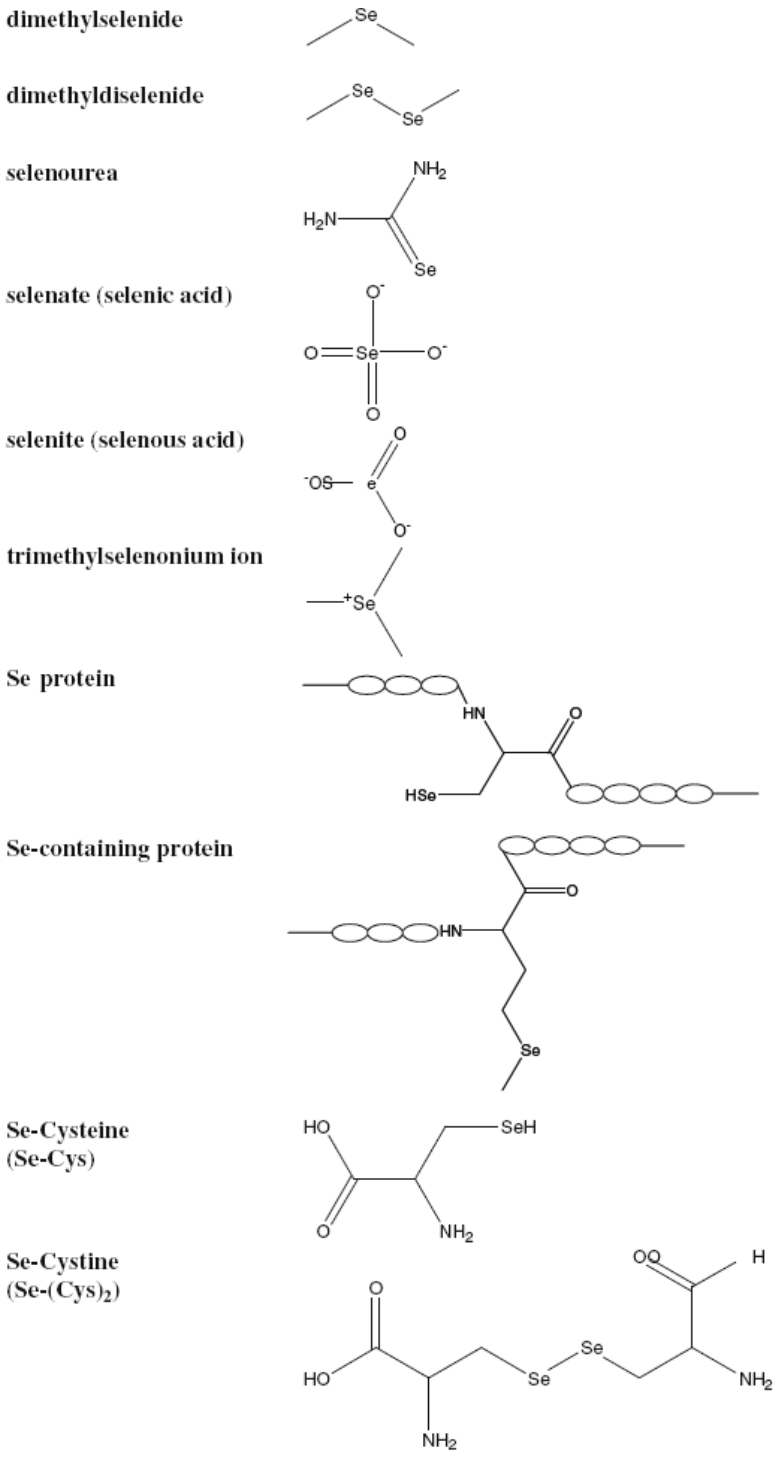


Figure 1-15 A : Selenium species from (Dumont *et al.*, 2006)

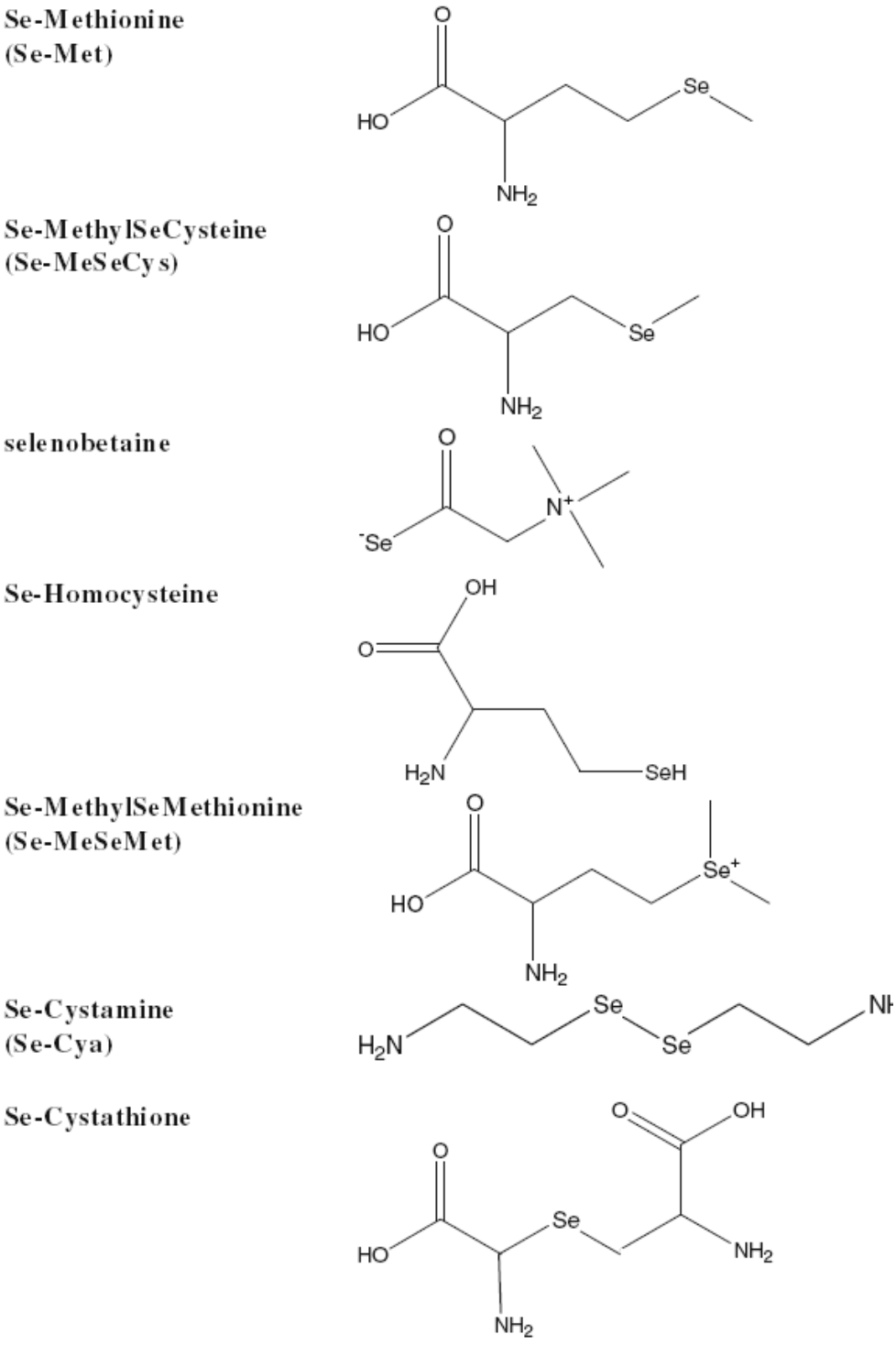


Figure 1-15 B : Selenium species from (Dumont *et al.*, 2006)

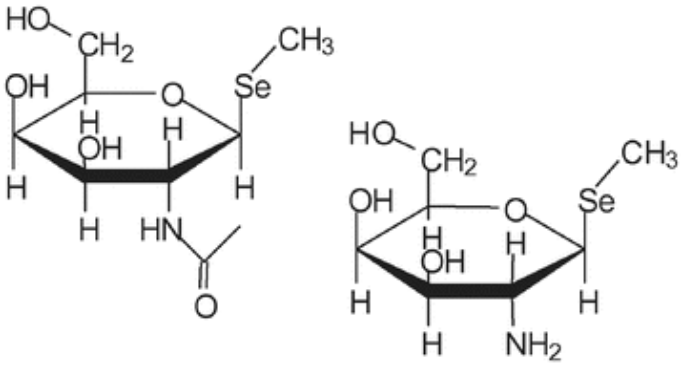


Figure 1-16: Se-methylseleno-N-acetylgalactosamine (SeGal-N-Ac) and Se-methylselenogalactosamine (SeGal-NH2) in human urine (Gammelgaard *et al.*, 2005).

Selenium can decrease the risk of some types of human cancers, especially for prostate, lung, and colon. According to mechanistic studies, the methylselenol metabolite pool has many desirable attributes of chemoprevention. It targets both cancer cells and vascular endothelial cells, whereas the hydrogen selenide pool in an excess of selenoprotein synthesis can lead to DNA single strand breaks, which may be mediated by some reactive oxygen species (Lü and Jiang, 2005). These authors propose a new paradigm based on which selenoproteins and specific Se metabolites account for cancer risk reduction or enhancement.

USEPA reports a NOEAL of 0.015 mg/kg/day for clinical Selenosis and a LOEAL of 0.023 mg/kg/day based on human epidemiological study (Yang *et al.*, 1989).

Selenium in the environment: Selenium is highly mobile under oxidizing, acidic, neutral and alkaline conditions, although its mobility decreases with decreasing pH (Gondi *et al.*, 1992) and it is immobile under reducing conditions. In aqueous solution, Selenium's chemistry is principally anionic, with selenite, SeO_3^{2-}, and selenate, SeO_4^{2-}, as the main forms present. Elemental Se, whilst relatively insoluble, is also stable over a wide pH range under reducing conditions (Brookins, 1988). The average crustal abundance of Se is 0.05–0.09 mg/kg and average concentrations in magmatic rocks rarely exceed these values.

Concentrations of Se in natural water rarely exceed 1 µg/L and average concentrations may be as low as 0.1 µg/L (Hem, 1992). During volcanic activity, Se escapes with high-temperature volcanic gases and its concentrations in volcanic rocks are therefore

generally low. Selenium is naturally present in soil with values ranging from very low concentrations (0.17 mg/kg – Keshan area in China) to very high concentrations (10-40 mg/kg Enshi province). In Europe, selenium soil concentration ranges between 0.03 and 29.70 mg/kg (Dumont *et al.*, 2006). Selenium concentration in soil is strongly associated with the content in the crops and in the food chain, and eventually to human intake. Cited examples of Se deficiency in China are thought to be a consequence of low dietary intake mainly as a result of poor content in soils.

Selenium in plants and food: It has been demonstrated that Se concentration in soil is not the most important factor limiting plant uptake, but rather the bioavailability of the chemical species. Not all plants are able to accumulate selenium. Rosefield and Beath (Rosenfield and Beath, 1964) classify plants in three categories in relation to their accumulation ability when growing in seleniferous soils: primary accumulators are able to grow well in soils highly enriched in Se (even higher than 1000 mg/kg); the secondary accumulators in a range on soil concentration between 50-100 mg/kg; and the non accumulator grows well in soil Se concentrations<10 mg/kg. Vegetals that naturally accumulate Se include brazil nuts, garlic (Allium) and genus Brassica (such as broccoli, Brussel sprouts, cabbage) (Dumont *et al.*, 2006). The tolerance to high Se concentration in soil has been explained with the formation of organo-selenium compounds that cannot be incorporated into proteins and the ability of these plants to volatilize selenium (Terry *et al.*, 2000).

Despite of its essentiality, selenium maximum content level in drinking water is fixed by a normative EU Directive 98/83 at 10 ppb. This example, clearly illustrates the fact that the border between essentiality and toxicity can be very narrow. Furthermore, the additive contribution from food cannot be ignored when calculating the actual internal dose for the general population.

Dietary intake and serum concentration of selenium can modulate arsenic toxicity in humans and have been found to decrease the risk of arsenic induced skin lesions (Chen *et al.*, 2007b). Walton *et al*. report that selenium as selenite is responsible for the inhibition of arsenite methylation in cultured hepatocytes and that inhibition may involve direct interactions between selenite and AsIII-methyltransferase (Walton *et al.*, 2003). A metabolic link between arsenic and selenium has been found with the identification of a compound of selenium, arsenic and glutathione (seleno-bis (S-glutathionyl) arsinium ion,

[$(GS)_2AsSe$]. Furthermore, $(GS)_2AsSe$ is found in the bile of rabbits treated with arsenite and selenite as a major arsenic and selenium excretory product (Gailer *et al.*, 2000a, Gailer *et al.*, 2002).

15.2 Copper

Copper (Cu) is an essential trace element, important for humans because it part of a number of metal enzymes including cytochrome C oxidase and superoxide dismutase that are required for collagen elastin formation and for catalyzing redox reactions (McClatchey, 1994). In the general population, food is the major source of copper intake. Particularly high concentrations are found in nuts (8 mg/kg), shellfish and offal (40 mg/kg) (Expert Group on Vitamins and Minerals, 2003). The Cu safe upper level established by the Expert Group on Vitamins and Minerals is 0.16 mg/kg bw/day (Expert Group on Vitamins and Minerals, 2003). Cu is absorbed from the small intestine by a mechanism shared with Zn; Cu is bound to binding proteins in the epithelium and then transported to the liver by albumin. Within 2 hours, 60-95% of copper is in the liver and in the hepatocytes where it is trapped and incorporated into proteins. Some Cu is incorporated into the structure of ceruloplasmin. Cu is distributed to the other tissues where it becomes part of the superoxide dismutase. The remaining copper is mainly excreted via the bile. Acute copper toxicity is rare and is caused by food or beverage contamination. Cu has emetic properties and an unpleasant taste that prevents accidental or deliberate ingestion. There are just a few available data on chronic copper toxicity in humans. Indian childhood cirrhosis (ICC) is a fatal disorder associated with accumulation of massive levels of copper in the liver. According to one hypothesis, ICC can be due to boiling and storing milk in copper containers. However, more recently the causative role of Cu has been dispelled (Sriramachari and Nayak, 2008). Isolated cases of idiopathic copper toxicosis (ICT) have also been reported in non-Indian communities in the US and Europe. Ford *et al.* (2000) found elevated serum copper concentrations to be associated with cardiovascular disease in US by analysing the Second National Health and Nutrition Examination Survey. More information on copper are presented in Chapter 4, section 1 of this thesis.

15.3 Zinc

Zinc (Zn) is essential because of biochemical, structural and functional reasons. It is involved in many processes as a cofactor of enzymes; it is a membrane stabilizer; and it is involved in anabolism and catabolism of carbohydrates, lipids, proteins and nucleic acids.

It is also essential in the transcription and translation of DNA. The principal clinical features of Zn deficiency are growth retardation, delay in skeletal and sexual maturation, impacts to mental health, dermatitis, diarrhoea and alopecia(Digirolamo and Ramirez-Zea, 2009), Zn overexposure causes nausea, vomiting, diarrhoea, fever and lethargy. Changes in lipid patterns and immune response have been associated with Zn supplementation. More information on zinc are presented in Chapter 4, section 1 of this thesis.

15.4 Manganese

Manganese (Mn) is an essential element in humans. Enzymes requiring Mn include manganese superoxide dismutase. Manganese metalloenzymes are involved in amino acid, cholesterol and carbohydrate metabolism (Shenkin, 2008).
The most relevant oxidative states from a biological and environmental point of view are Mn^{2+}, Mn^{4+} or Mn^{7+} (US EPA, 1994).

The estimated daily requirement for Mn is 30-50 μg/kg (Gerard and Combes, 2005). The Institute of Medicine of the National Acedemies (IOM) sets an adequate daily intake (AI) for Mn at 3 μg/day for infants younger than 7 months (IOM, 2001).
Absorption rate is influenced by intake, chemical form, and by synergists or antagonists such as Fe and Cu. Oral absorption is regulated by homeostasis; uptake is regulated so that when dietary Mn levels are high, the gastrointestinal absorption is reduced. It is quite low in adults (1-5%) (Ljung and Vahter, 2007) but is higher in infants because about 20% of the manganese in formula-fed infants is absorbed. The main excretion route is through the biliary system.

According to WHO, possible indicators of manganese exposure can be derived from the analysis of blood and hair. However, Mn levels in blood do not provide data on long-term exposure with background levels (WHO, 2004).

Mn is normally present in food and for this reason its deficiency in humans is rare. Main food sources include leafy vegetables, nuts, grains and animal products (IOM, 2001). Nut groups have a content of 24.9 mg Mn /kg. Other foods show a range of concentrations has been found in other food groups: the lowest concentration is found in milk (0.022 mg/kg) and the highest in bread (8.01 mg/kg) in the UK Total Diet Study (FSA, January 2009). Even though food is thought to be the most important source of manganese exposure in

the general population (ATSDR, 2000; US EPA, 2002), manganese can occur naturally in many surface water and groundwater sources such as in Bangladesh (Frisbie *et al.*, 2009) and Cambodia (Luu *et al.*, 2009).

Neurotoxicity is a known effect of long-term exposure to inhaled manganese in humans and animals, but the potential for neurotoxicity resulting from oral exposure is less well characterized and just a few reports exist on the topics for human exposure. Rodents are not considered to be a good experimental model (EPA, 1996).

Adverse human effects reported from overexposure *via* dusts or fumes include "Manganism": this has a "Parkinson-like syndrome," including weakness, anorexia, muscle pain, apathy, slow speech, monotonous tone of voice, emotionless "masklike" facial expression and slow, clumsy movement of the limbs. In general, these effects are irreversible.

Some epidemiological studies report adverse effects in humans through drinking water. In Japan, as a result of a massive industrial contamination, human exposure to high levels of Mn (as much as 28 mg/L), symptoms including lethargy, increased muscle tone, tremor, mental disturbances and in some cases death, occurred (Kawamura *et al.*, 1941). In Greece, progressive increases of Mn in drinking-water (3.6–14.6 µg/L in the control area and 81–253 µg/L and 1800–2300 µg/L) was associated with a progressively higher prevalence of neurological effects in elderly people (Kondakis *et al.*, 1989).

Another study of the relationship between decreased intellectual functioning and manganese exposure through drinking water is reported by (Wasserman *et al.*, 2006) for a population of Araihazar (Bangladesh).

The hazard posed by overexposure to manganese must be weighed against the necessity for some minimum amount of manganese in the diet, because manganese is an essential nutrient, acting as a component of several enzymes and a participant in a number of important physiological processes.

The IOM sets a tolerable upper intake level of 11 mg/day for adults. Evaluations of standard diets from the USA, the United Kingdom and the Netherlands reveal average daily intakes of 2.3–8.8 mg of manganese per day (WHO, 2004). Depending on individual

diets, however, a normal intake may be well over 10 mg of manganese per day (Schroeder *et al.*, 1966), especially for vegetarian diets.

For drinking water, WHO established a health-based guideline value of 0.4 mg/L as adequate to protect public health, assessing a concentration below 0.05 mg/L as acceptable for consumers, although this may vary with local circumstances. The European Council (Directive 98/83/EC: The Drinking Water Directive) (Conuncil of the European Union, 1998) therefore set a parametric value for Mn of 50ppb. However, Ljung and Vahter (2007) highlighted the need for re-evaluation of Guideline Values by WHO and consequently the limit set by the Drinking Water Directive, emphasizing that although a lower value may not necessarily be required for the majority of the population, some sensitive groups, such as infants and elderly, may be at risk with the present guideline value for drinking water.

15.5 Cobalt

Cobalt (Co) is an essential element because it is required for the production of vitamin B12 or cyanocobalamin. Cobalt is a naturally-occurring element that has properties similar to those of iron and nickel. It exists in two valence states: Co(II) and Co(III); Co (II) is often used in the chemical industry whereas metallic Co is used in the production of alloys, especially "hard metals" (Barceloux, 1999). Elemental cobalt is a hard, silvery grey metal (Hultman, 2007). The concentration of cobalt in soil varies widely, with a range of 1- 40 ppm and an average level of 7 ppm (EPA, 2004). Soils containing less than about 3 ppm of cobalt are considered cobalt-deficient because plants growing in them do not have sufficient cobalt to meet the dietary requirements of cattle and sheep (EPA, 2004). On the other hand, soils near cobalt-containing mineral deposits, mining and smelting plants may contain much higher levels of cobalt. Air contains very small amounts of cobalt, < 2 ng/m^3 (EPA, 2004). Surface and ground waters in the United States have generally low cobalt concentrations (1-10 ppb) (EPA, 2004). In most drinking water, cobalt levels are less than 1-2 ppb (EPA, 2004).

For the general population, food (mainly meat and dairy) is the largest source of cobalt intake (EPA, 2004). The recommended daily intake of vitamin B_{12} is 6 µg and the average person consumes about 11 µg of cobalt a day from their diet (EPA, 2004).

Occupational exposure to cobalt results in serious effects on the lungs, including asthma, pneumonia, and wheezing, at levels of 0.005 mg cobalt/m^3 while working with a cobalt-tungsten carbide alloy (EPA, 2004). People exposed to 0.007 mg cobalt/m^3 developed allergies to cobalt that result in asthma and skin rashes (EPA, 2004).

Based on animal data, the IARC has determined that cobalt is possibly carcinogenic to humans (IARC, 2006).

Immunological reactions to Co is mainly sensitization. It is quite unusual in the general population (<1%), but may occur concomitantly with sensitization to Ni in women and Cr in men (Hultman, 2007).

The most common hypersensitivity reaction to Co is allergic contact dermatitis, a type IV reaction (Garner, 2004), although occupational asthma with increased serum IgE antibodies to Co, indicative of a type I reaction, has also been reported (Shirakawa and Morimoto, 1997).

15.6 Nickel

Nickel (Ni) is the 24th most abundant element in the Earth's crust. Ni occurs naturally in soils and volcanic dust and emitted species into the atmosphere depend on the source. Background levels of nickel in soils vary in relation to local geology and anthropogenic inputs, but concentrations typically range between 4 and 80 ppm (ATSDR, 2005).

Ni is a metalloid with unique physical and chemical properties that mean it is widely used in modern industry. The high use of nickel-containing products results in environmental pollution of Ni. Human exposure to nickel might occur via inhalation and ingestion. Nickel is not identified as essential for the human body, it has only adverse effects on humans. IARC evaluated all nickel compounds as carcinogenic for humans except from metallic nickel (IARC, 1990). Cancer of the lungs and nose is reported in occupationally exposed groups (mining and smelting). However the molecular basis for nickel carcinogenicity has not yet been proved because nickel compounds are weekly mutagenic. Nickel produces oxidative stress depleting glutathione, activating NF-kB[7] and other transcription factors.

[7] proteins comprise a family of structurally-related eukaryotic transcription factors that are involved in the control of a large number of normal cellular and organismal processes, such as immune and inflammatory responses, developmental processes, cellular growth, and apoptosis

Nickel induces contact dermatitis in exposed people sensitive to nickel, though nickel is in fact used for alloys in the production of jewellery and prosthesis.

About 20–35% of the inhaled nickel is absorbed into the blood. Absorption of nickel following oral exposure varies (3–40%), with a greater bioavailability from water than food. Most of the absorbed nickel is excreted in the urine (ATSDR, 2005).

Table 1-10: Main parameters related to human consumption/toxicity/carcinogenicity of trace elements

	Biochemical or Physiological function (*)	**RDIs [mg/ day]**	**UL [mg/ day]**	**Essentiality (=)**	**Adverse effect from over exposure (*)**	**IARC (&)**	**EU MCL [µg/L] (+)**
As	No biological function in humans	-	ND	Potentially toxic	arsenicosis, cancer	**1**	**10**
Cr	Help in glucose homeostasis in blood	0.020 - 0.044	N D	Essential	Chronic renal failure	**3** (Cr III) **1** (Cr VI)	**50**
Cu	Component of enzymes in Fe metabolism	0.7-1.3	10	Essential	Gastro-intestinal distress, liver damage	-	**50**
F	Inhibits dental caries and stimulate bone formation	2-4	10	Potentially toxic	Fluorosis	3	**1500**
I	Component of thyroid hormones; prevents goiter	0.12-0.29	1.1	Essential	Elevated TSH concentration, deficiency if I linked to cretinism in Sicily	-	
Fe	Component of Hemoglobin;prevent microcytic hypochromism anemia	8-27	45	Essential	Gastro-intestinal distress	1 (founding)	**200**
Mg	Enzyme cofactor	240-420	350	Possibly essential		**-**	
Mn	Involved in bone formation, coenzyme in metabolism of amino-acids, cholesterol, carbohydrates.	1.6-2.6	11	Probably essential	Manganism, Neurotoxicity	**-**	**50**

	Biochemical or Physiological function (*)	**RDIs [mg/ day]**	**UL [mg/ day]**	**Essentiality (=)**	**Adverse effect from over exposure (*)**	**IARC (&)**	**EU MCL [µg/L] (+)**
Mo	Cofactor for enzymes involved in catabolism of sulphur amino acids, purines and pyridines.	0.034 - 0.045	2	Essential	Reproductive effects in animals	-	
Ni	No clear biological function in humans. It may serve as a cofactor of metalloenzymes and facilitate iron absorption or metabolism in microorganisms.	ND	1	Possibly essential		**2B**	**20**
Si	No biological function in humans has been identified. Involved in bone function in animal studies.	ND	ND	Possibly essential	None reported	-	
Se	Defence against oxidative stress and regulation of thyroid hormone action, and the reduction and oxidation status of vitamin C and other molecules	0.04-0.07	0.4	Essential	Hair brittleness and loss	3	**10**
Zn	Enzymes cofactor, involved in transcription and translation of DNA	8-12	40	Essential		-	

(*)Values and information from Food and Nutritional Board, table accessed from http://www.nap.edu/catalog.php?record_id=10026 (DRI=Dietary Reference Intakes, reported range includes both female and male adults, pregnancy and lactation; UL= the maximum level of daily nutrient intake that is likely to pose no risk of adverse effects.);(&) from IARC (2008) (Group 1: Carcinogenic to humans; Group 2A: Probably carcinogenic to humans; Group 2B: Possibly carcinogenic to humans; Group 3: Not classifiable as carcinogenic to humans; Group 4: Probably not carcinogenic to humans);(+) from EU Directive 98/83 ;(=) WHO, 1996.

16. Risk assessment for trace elements

An approach to estimate the acceptable daily intake for essential and non essential trace elements is the use of Risk Assessment (RA). RA is defined as a four step, scientific process

meant to address the relationships between exposure to a substance and the likelihood of adverse effects/adverse health effects in exposed sub/populations. RA based approaches classically involve the following steps: hazard identification, hazard characterization, exposure assessment and risk characterization (WHO, 1999, WHO, 2006).

Toxicokinetic and toxicodynamic factors are taken into account when formulating an RA for toxic metals and trace elements (Yokel *et al.*, 2006). Pharmacokinetics based models (PKBM) and biologically based models can represent valuable tools for the identification of target organs and for risk assessment (Kenyon *et al.*, 2008). The quantitative outcome of the hazard characterization step of an RA, is the specification of an Upper Level of Intake (UL), which is formally defined as the maximum level of habitual intake from all sources of a nutrient or related substance judged to be unlikely to lead to adverse health effects in humans.

The usual method for obtaining a threshold of effect is to divide the NOAEL or the LOAEL by an Uncertain Risk factor (UF):

Acceptable Daily Intake or water quality standard,etc. = NOAEL / (UFs) (Equation1)

The UF is defined as the product of different components of the uncertainty, and needs to be considered in the extrapolation from animal studies to human relevance, trying to include effects on a sensitive subset of the general population. A possible definition of UF is:

$$UF = (UFa * UFh * UFt * UFd) \quad \text{(Equation2)}$$

Where:

UFa= uncertainty from animal data to human
UFh= uncertainty from human variability
UFt= temporal factor
UFd=uncertainty due to data gap

A typical UF is 100. However, in the case of RA for essential trace elements, this approach could cause the definition of a UL below the necessary dose for health and well-being. For this reason, Joint FAO/WHO Expert Committee on Food Additives (JECFA) determined that in the case of nutrients, the use of a lower safety factor may be appropriate in order to result in a level of nutrient intake high enough to satisfy nutritional needs and to maintain health.

Another way to estimate a threshold is the calculation of the Benchmark dose, which is used by U.S. Environmental Protection Agency (USEPA).

When studying the exposure to geogenic trace elements that have multiple sources of exposure in the environment, the approach developed by JECFA (JECFA, 2005) for risk assessment of nutrients can be useful. JECFA model outlines the need for including dietary intake assessments in the procedures required for risk characterization.

Considering what has been stated so far, some key points in the evaluation of acceptable range of intakes for trace elements are the following:

1. Multiple sources of exposure to trace elements are present in the environment such as food and drinking water. Therefore the calculation of the actual intake from GI tract has to take into account at least these two main components.
2. Some trace elements are essential but some are not and this makes a difference in the approach for the evaluation of Maximum Content Level (MCL) for normative regulations in drinking-water and food, the estimation of permissible human exposure and the Upper Level of Intakes.
3. It is important to assess the intake of certain trace elements from food prior to the estimation of the cumulative daily exposure (JECFA approach). Total Diet Studies (TDS) that are continuous market basket-type surveys, in which foods representing the average diet are purchased, prepared and combined into groups of similar foods for analysis, represent useful tools to estimate the food contribution to trace elements intake.
4. A risk assessment approach is a valuable tool in the estimation of ULs for trace elements. It should consider toxicokinetic and toxicodynamic factors in setting exposure standards.

17. Aims of the thesis

The aims of this thesis are as follows:

1. To evaluate the impact of a diet high in rice consumption on urinary arsenic profile of two ethnic groups in the United Kingdom (Chapter 3).

2. To evaluate the possible influence of ethnicity and diet in volunteers from the United Kingdom by monitoring urinary total trace element profiles for arsenic, selenium, zinc and copper (Chapter 4).

3. To apply an "-omics" approach to evaluate changes in the human urinary metabolomic profile as a function of arsenic exposure and other factors (Chapter 5) in an exposed population from Bangladesh and a UK based Bangladeshi population.

4. To study the relationship between neurological diseases and trace elements with a specific focus on a multiple sclerosis cluster reported in the Mt. Etna region in Sicily, Italy (Chapter 6).

5. To determine the content of a range of trace elements in the cerebrospinal fluid of multiple sclerosis patients and controls, in order to detect possible alterations connected to disease and disease progression (Chapter 7).

A highly multidisciplinary methodology has been applied in order to address these research questions. Analytical techniques used to detect biomarkers of exposure and candidate biomarkers of biochemical effect include Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and High Pressure Liquid Chromatography ICP- MS (HPLC-ICP-MS) for determination of trace element in bio fluids and Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H-NRM) for urinary metabolomics. The multidisciplinary methodology used in this thesis is discussed in detail in Chapter 2.

Chapter 2

HUMAN BIOMONITORING AND METHODOLOGY

Human bio-monitoring (HBM) is "a systematic continuous or repetitive activity of collection of biological samples for analysis of concentrations of pollutants, metabolites or specific non-adverse biological effect parameters for immediate application, with the objective to assess exposure and health risk to exposed subjects, comparing the data observed with the reference level and — if necessary — leading to corrective actions" (Zielhuis, 1984).

In order to explore human exposure to geogenic/anthropogenic trace elements, human bio-monitoring studies are required. In this sense, HBM therefore consists of the analytical measurement of body burden of toxic chemical compounds, elements or their metabolites in biological samples such as urine or blood. It can be performed on the general population or on selected groups of people (as in Chapters 3 and 4), those who are occupationally exposed, or those suffering from a specific pathology (Chapters 6 and 7).

The outcome of bio-monitoring studies are precious pieces of information for policy making, providing data on safe levels of exposure to a certain environmental agent (WHO, 1999, WHO, 2006) and helping to create and maintain a healthy environment. Bio-monitoring studies can also be aimed at elucidating the role of a certain type of diet or therapy in the induction of changes in metabolites in biofluids. Bio-monitoring of biofluids

in sufferers from certain pathologies leads to the discovery of new prognostic and diagnostic biomarkers of the diseases. HBM studies represent powerful and challenging ways to answer questions about the state of health of human beings.

Even if extremely useful, the monitoring of human beings poses some problems. Setting up a HBM study is not as straightforward as setting up a batch experiment; external conditions can be extremely variable from one subject to another. For instance, even in the same city or in the same family, each individual is exposed to a different extent to environmental contaminants as a result of personal habits, diet, cultural practice, occupation and daily routine. Such factors act as confounders. Nevertheless, if such factors are correctly reported and taken into account , it is possible to define the *individual environment* for rationalization and interpretation of laboratory data. In this sense, Chapter 3 explores how diet can alter the daily intake of rice in people sharing the same geographical environment as a result of ethnicity. Chapter 4 explores this problem for a greater group of ethnicities living in the UK and for a greater number of trace elements (arsenic, selenium, copper and zinc).

HBM studies can be generally differentiated into 3 subcategories: dose monitoring, biochemical effect monitoring and biological effect monitoring (Angerer *et al.*, 2006, Angerer *et al.*, 2007)

- **Dose monitoring** is the determination of hazardous substances or their metabolites in body fluids.
- **Biochemical effect monitoring** is the quantification of the reaction products of reactive substances with biological molecules such as DNA or proteins.
- **Biological effect monitoring** is related to the measurement of early biological effects caused by chemical substances, for instance sister chromatid exchange rates, micronuclei, enzyme activities.

Non invasive sampling procedures, stability of samples and the presence of a reliable and low cost analysis approaches are desirable features for large scale human biomonitoring studies on the general population.

Figure 2-1 shows the properties of different, non-invasively collected samples for human biomonitoring.

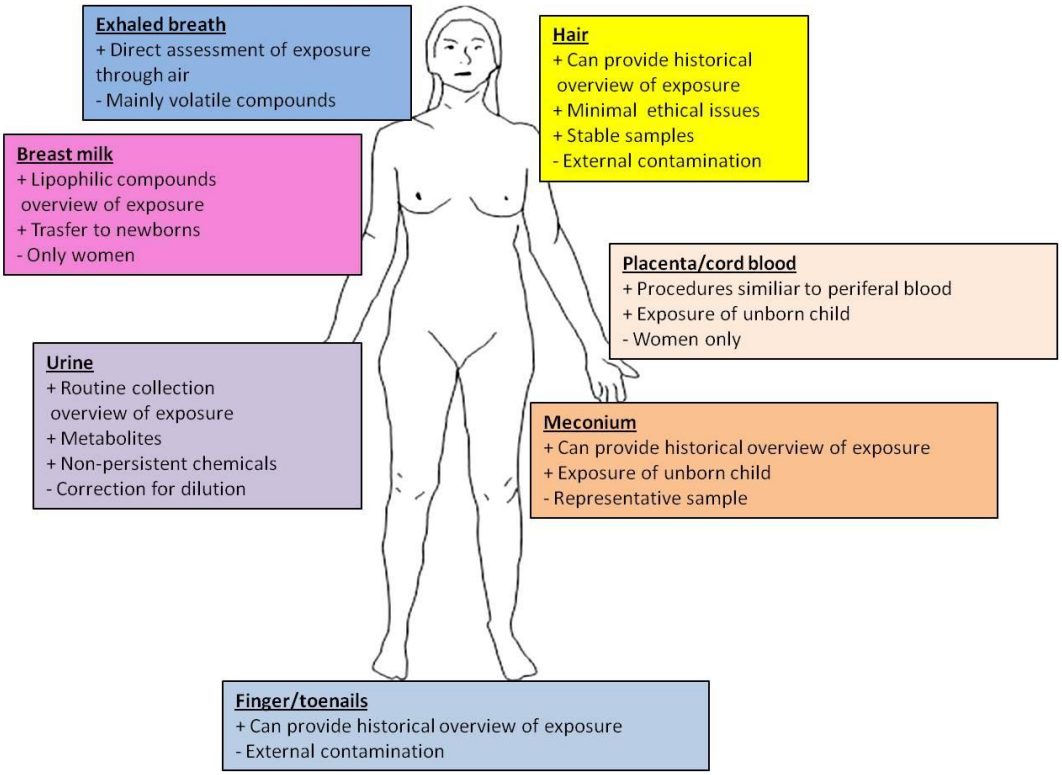


Figure 2-1: Some properties of different non-invasively collected matrices for routine human biomonitoring application from Smolders *et al.* (2009).

Chapters 3, 4, 6 and 7 represent dose monitoring studies assessing the presence of arsenic and other trace elements in human biofluids and tissues. Chapter 5, is a study to explore changes in urine in relation to arsenic and other trace elements in order to monitor the biochemical and biological effect.

1. Human biomonitoring for assessing trace elements exposure in Europe and Other Regions of the World

Among the human bio-monitoring studies carried out in Europe for assessing trace element baseline levels in the general population, the EURO TERVIHT project (Trace Element Reference Values in Human Tissues) (Sabbioni *et al.,* 2002) was carried out in the 90's. It aimed to establish and compare trace element reference values in tissues from inhabitants of the European countries as baseline values for clinical/toxicological assessment studies (Minoia *et al.*, 1994; Sabbioni *et al.*, 1992; Minoia *et al.*, 1990). It reviewed/published data on trace elemen baseline levels in urine, serum and other

biofluids in Danish (Iversen *et al.*, 1998, Poulsen *et al.*, 1994), Czech and Slovak (Kucera *et al.*, 1995), Belgian (Cornelis *et al.*, 1994), British (Hamilton *et al.*, 1994b) and Italian (Sabbioni *et al.*, 1992) populations, giving a first insight into the problem. Unfortunately, this monitoring activity is not carried out on a regular basis by any European Agency or surveillance organisation. At a national level, detailed studies have been carried out in Italy by the Istituto Superiore di Sanità (Alimonti *et al.*, 2010), reporting trace elements in blood, urine, nails, CSF and plasma values for the general Italian population. In Germany, studies by Heitland *et al.* provided information on levels of trace elements in urine of children and adults (Heitland and Kolster, 2008; Heitland and Köster, 2006). Very little has been published in the UK (Morton and Mason, 2006).

In the USA, data from the National Health and Nutrition Examination Survey (NHANES), which is carried out on a regular basis, are available for certain trace elements in urine and other biosamples of the general population and people affected by certain diseases (Caldwell *et al.*, 2009; Navas-Acien *et al.*, 2008).

Most of the human biomonitoring studies carried outside of Europe and USA have focused on exposed populations living in highly contaminated areas, like in Bangladesh (Lindberg *et al.*, 2008) , Cambodia (Gault *et al.*, 2008) and China (Yu *et al.*, 2007) rather than for general monitoring purposes.

2. Background to study populations monitored in this thesis

2.1 Definition of Ethnicity

The word ethnicity derives from the Greek word *ethnos*, which means nation. Ethnicity is the social group a person belongs to, and either identifies with or is identified with by others, as a result of a mix of cultural and other factors including language, diet, religion, ancestry, and physical features traditionally associated with race (Bhopal, 2004). As stated by Bhopal *"Ethnicity is a multi-faceted quality that refers to the group to which people belong, and/or are perceived to belong, as a result of certain shared characteristics, including geographical and ancestral origins, but particularly cultural traditions"* (Bhopal, 2004). Ethnicity is not fixed and easily measurable and it is different from race, nationality and religion, although it can include these concepts.

In this piece of work, the different ethnic groups have been defined as in (Bhopal, 2004):

"Caucasians: an Indo-European group. This is Blumenbach's 18th century term for the white race of mankind, which he derived from the people who lived in the Caucasus. This term is usually used synonymously with Caucasoid, European, or White".

"Bangladeshi: a person whose ancestry lies in the Indian subcontinent who self identifies, or is identified, as Bangladeshi. Between 1947 and 1971 the land known as Bangladesh was East Pakistan and before that India. There is no clear cut equivalent in terms of racial classifications, though historically Northern Indians have been classified as white, and some Indian tribes as aboriginal."

"Indian: a person whose ancestry lies in the Indian sub-continent who identifies, or is identified, as Indian."

"Pakistani: a person whose ancestry lies in the Indian subcontinent who identifies, or is identified, as Pakistani. Some Pakistanis may have birth or ancestral roots in the current territory of India but identify with Pakistan, a country created in 1947."

2.2 Definition of Race

According to the pure biological concept of race, human populations are divided into sub-species mainly on the basis of visible physical characteristics (such as skin colour and hair texture, which reflect ancestry and geographical origins). This view was dominant from the early 19th century and it was further promoted by the Nazis at the end of the second world war (Senior and Bhopal, 1994).

The modern concept of race has a social origin rather than its biological basis. Race is a way of defining, for social purposes, populations that look different and with different ancestral roots. Even this social concept of race is ultimately based on physical factors and hence biological factors (Bhopal, 2004).

2.3 Population of England and Wales

In the middle of 2009 England and Wales consisted of 54,809,100 people. The greatest part of this population is represented by White British (see Table 2-1). There is a growing component of non-White British populating the area. Of these, the most populous communities are the Indians and Pakistanis. The greatest part of the non-White British group is settled in London (40.5%); however the Midlands host a considerable proportion (30% including both East and West). Notable concentrations of non-White British

communities include Leicester (highest proportion of Asian Indian at 19 percent); and Bradford (where 13 percent are Asian Pakistani). 21 percent of the Tower Hamlets population is estimated as Asian Bangladeshi – with the next highest proportion being 8 percent in neighbouring Newham. While Tower Hamlets makes up only 0.4 percent of the total population of England and Wales, it is estimated to contain more than 12 percent of the Asian Bangladeshi group.

In this piece of work, a first study group from the UK is monitored in Chapter 3, focusing on the effect of a rice based diet on urinary arsenicals. In this case a UK Bangladeshi group was compared to a white Caucasian group. In Chapter 4, four main ethnicities (Bangladeshi, Indian, Pakistani and White) are compared in terms of trace element urinary profile.

2.4 Inequality of health in the UK

Evidences of inequalities in health in ethnic minority communities are reported for people living in the UK in the Health Survey of England carried out in 1999 (NHS, 2004). All South Asian groups living in the UK show a higher rate than the general population of cardiovascular disease conditions, including angina, heart attack, stroke, irregular heart rhythm and diabetes.

Both Pakistanis and Bangladeshis show higher rates of these conditions than Indians. In particular, for diabetes, Pakistanis and Bangladeshis of both sexes showed rates over five times higher than the general population and Indians almost three times higher (Primatesta and Brookes, 1999).

Furthermore, British Asians have increased levels of plasma glucose and glycated haemoglobin (HbA1c), fasting insulin, and triglyceride concentrations, higher levels of adiposity (particularly central adiposity) and lower HDL-cholesterol concentrations (Barba *et al*., 2004; McKeigue *et al*., 1991; Fagot-Campagna *et al.,* 2000; Kuh and Ben-Shlomo, 1997). Among South Asians living in the UK, diabetes prevalence tends to be higher among UK Bangladeshis and Pakistanis compared to Indians (McKeigue *et al.,* 1991; Kuh and Ben-Shlomo, 1997; Bhopal *et al.,* 1999).

Table 2-1: Population of England & Wales in 2009 from (Office of National Statistics, 2011)

Ethnic group	Thousands of People
	54,809.1
White:British	**45,682.1**
White:Irish	574.2
White:other white	1,932.6
Mixed: White and Black Caribbean	310.6
Mixed: White and Black African	131.8
Mixed: White and Asian	301.6
Mixed: other mixed	242.6
Asian: Indian	1,434.2
Asian: Pakistani	1,007.4
Asian: Bangladeshi	392.2
Other Asian	385.7
Black Caribbean	615.2
Black African	798.8
Other Black	126.1
Chinese	451.5
Other	422.6
Non-'White British'	**9,127.1**

2.5 Population of Sicily

The Island of Sicily is the biggest region of Italy. According to the last census (ISTAT, 2007) more than 5 million people live on Sicily, which is 8.5% of the total Italian population. Foreigners represent just 1.6% of the population of Sicily.

Sicily is organised in 9 provinces, of which the most populous are Palermo (1,241,241 inhabitants in 2006), Catania (1,076,972) and Messina (653,861), which collectively host about 60% of the entire Sicilian population (ISTAT, 2007).

2.6 Health status in the Mt. Etna Region

The Eastern part of Sicily hosts Mt. Etna, which is the highest active European volcano (see Figure 2-2). Volcanic activity is monitored daily by the National Institute of volcanology (http://www.ct.ingv.it/index.php). Several reports and papers document the presence of levels of trace elements occurring in groundwaters (Aiuppa *et al.*, 2003, Giammanco *et al.*, 1996) and plume (Quayle *et al.*, 2010). The introduction section of Chapter 6 reports more

data on this issue. Figure2-2 A shows the volcano, the city of Catania and lava and ashes emissions.

Numerous urban settlements are present in the surroundings of Mt. Etna. The frequent emissions from the plume from the top part of the volcano are spread on Catania and other cities. People from Mt. Etna consume groundwater (see Figure 2-3 A), collecting it from fountains and wells. Furthermore, the public distribution of drinking water in households relies entirely on groundwater.

It is well known from historical sources that the city of Catania has been destroyed several times (at least 7) from earthquakes and lava flows deriving from Mt. Etna (see Figure 2-3 B). Additionally, the health risk and effects related to chronic exposure to the 'volcanic environment' has not been fully investigated in this area.

A very high incidence of malignant pleural mesothelioma has been found in the area city of Biancavilla in the Mt. Etna region. Fibrous amphiboles (see Figure 2-4) of volcanic origin were found in products from local quarries, used for years to build houses (Bruni et al., 2006). Samples from three sites in the area were characterized and an abundant presence of mineral fibres was found. Fibrous amphiboles were found in building materials and airborne particulates sampled in urban sites with high dust emissions due mainly to unpaved roads. Moreover, amphibole fibres were detected in the lung tissue of a woman who died of pleural mesothelioma. According to the authors (Bruni et al., 2006), the amphibole fibre diffusion in the Biancavilla environment lasted for many years and had been at its maximum during the sixties and the seventies because of the uncontrolled development of the local building industry. However, according to the authors, the environmental risk of exposure is decreased thanks to both the closing of the stone quarries and the urbanization works with asphalting of dusty roads.

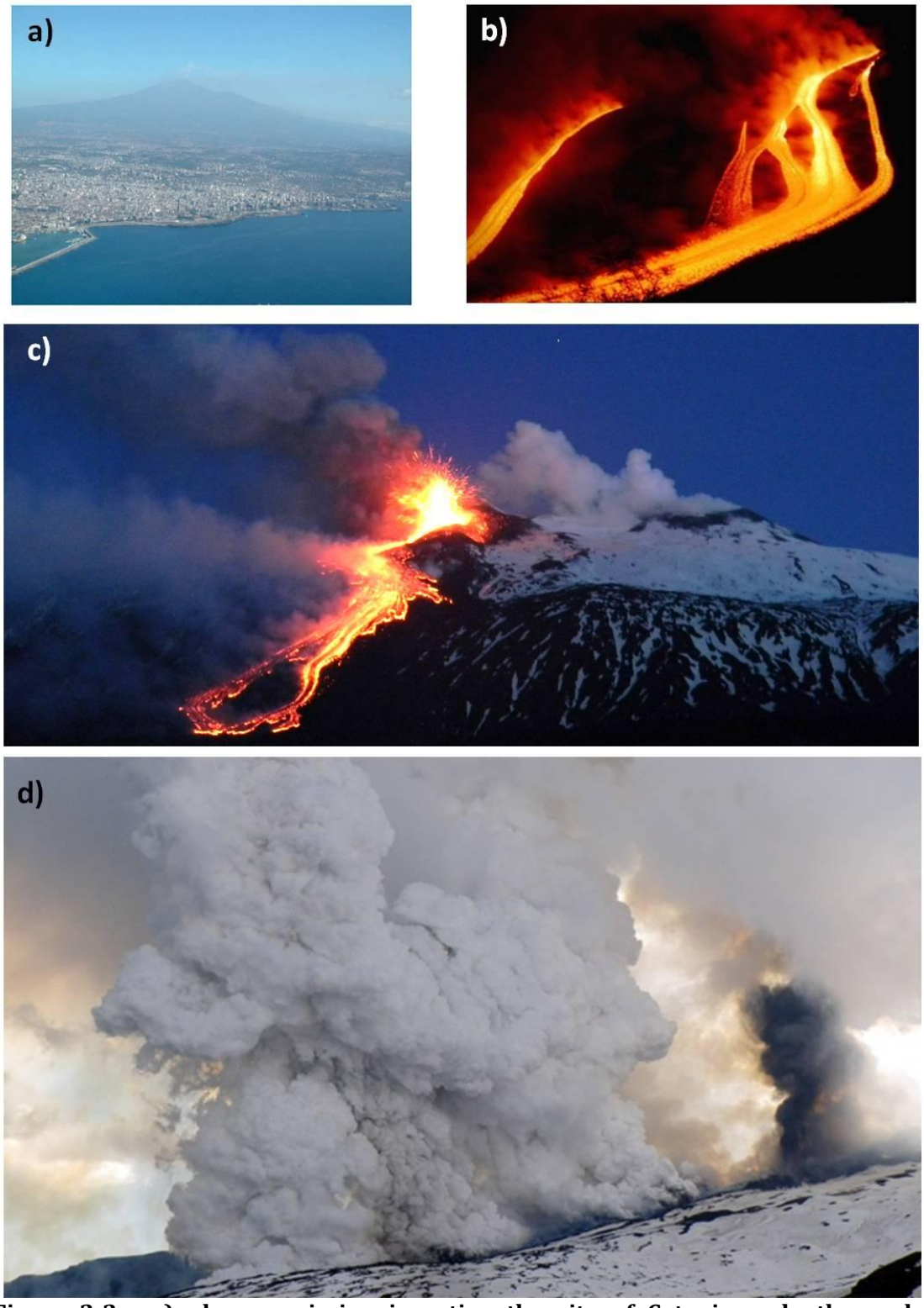


Figure 2-2 : a) plume emission investing the city of Catania and other urban settlements; b) eruption and fluid lava emission (1971,by Giuseppe Scarpinati); c) final stage of emission of 12th May 2011 from Monte Fontane, East flank (by Boris Behncke); d) lava flow on the snow and strong explosions and pyroclastic flows (10th April 2011, by Francesco Ciancitto, INGV-CT)

Figure 2-3: a) the habit of collecting water from fountains and wells is widespread in the population from Mt. Etna, Currune, Biancavilla (2007, by C. Cascio) b) fresco documenting the eruption of 1669 that reached the city of Catania and the sea (Giacinto Platania, 1675, Cathedral of Catania).

Exposure to volcanic ashes can have respiratory health effects. A review on published clinical, epidemiological and toxicological studies by Horwell (Horwell and Baxter, 2006) highlights the complexity of evaluating the long-term health risk (silicosis, non-specific pneumoconiosis and chronic obstructive pulmonary disease) in populations from prolonged exposure to ash due to persistent eruptive activity. Collection of published data from different volcanic sites shows that acute and chronic health effects of volcanic ash depend upon particle size (proportion of respirable-sized material), mineralogical composition (i.e. crystalline silica content) and the physicochemical properties of the surfaces of the ash particles (Horwell and Baxter, 2006). The incidence of acute respiratory symptoms (e.g. asthma, bronchitis) varies greatly after ashfalls, from very few to population-wide outbreaks of asthma. However data from Mt. Etna are not reported in this study from 2005.

A recent study of Mt. Etna particulate (Barsotti et al., 2010) computes, by numerical simulations, the likelihoods of experiencing critical 10-μm volcanic particle (VP_{10}) concentrations in ambient air and tephra ground deposition at various populated locations around the volcano, including the city of Catania and at key infrastructures, such as airports and main roads. Results show how the towns and infrastructures on the Eastern side of the volcano are significantly more exposed to ash-related hazards than those on the west side, in accordance with wind statistics. The use of a simple re-mobilization model highlights the fact that particle re-suspension needs to be considered in the estimation of VP_{10}values, showing how the potential importance of such a process in producing airborne ash concentrations is potentially dangerous for human health (Barsotti et al., 2010).

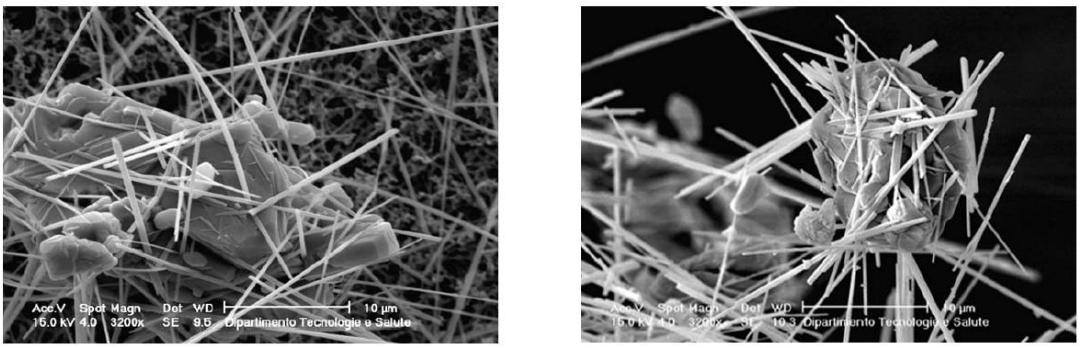


Figure 2-4: Amphibole fibers detected in Biancavilla (Mt. Etna region) from (Bruni et al., 2006)

During Autumn 2002, a large eruption of Mount Etna occurred. It lasted for 3 months, showing peaks of intense volcanic activity characterised by explosions, jets hundreds of metres high and seismic activity. Volcanic ashes were carried for kilometres, causing discomfort to the population of Catania and other inhabited areas near the volcano. Fano (Fano *et al.*, 2010) (Fano *et al.*, 2005) evaluated the short term effect of this eruption: mortality and hospital admissions data were collected for all residents of the city of Catania during both eruption and non-eruption days in 2002, using the corresponding days in 2001 as a control. Relative risks (RR) of health events during the eruption with respect to the control period were computed both for mortality and for hospital admissions. No changes in all cause (RR 0.98, 95% CI 0.86–1.12) and cardiovascular (RR 1.09, 95% CI 0.90–1.32) mortality were observed, whereas a significant decrease in mortality for respiratory diseases during the eruption period was detected (RR 0.46, 95% CI 0.46–0.82). However, hospital admissions for cardiovascular diseases significantly increased (RR 1.18, 95% CI 1.08–1.29), particularly for ischemic heart diseases (RR 1.31, 95% CI 1.10–1.56) and for cerebrovascular diseases (RR 1.24, 95% CI 1.05–1.47), especially among people aged 65 years and over. Notable, but not statistically significant, were admissions for eye disorders, which were two times higher during the eruption period than in the previous year. However, a decrease in hospitalisations for trauma was observed (RR 0.9, 95% CI 0.8–1.0). No statistically significant association appeared between daily PM10 levels and daily hospital admissions, although the effect increased with time lags suggesting a delayed effect on cardiovascular health. The relationship between daily PM10 level and cardiovascular admissions following Mount Etna's eruption shows that the effect was not immediate but, rather, delayed. The author (Fano *et al.*, 2010) explains the temporary increase in cardiovascular morbidity, observed particularly among the elderly, as being a consequence of acute stress.

Pellegriti *et al*. (Pellegriti *et al.,* 2009) reported a significant increase in the incidence of papillary thyroid cancer in Catania. Furthermore, patients in Catania more frequently showed the BRAF V600E gene mutation than patients elsewhere in Sicily. Cancer incidence was statistically significantly lower in rural areas than in urban areas of Sicily (P =0.003). No association with mild iodine deficiency or industrial installations was found. The authors (Pellegriti *et al.,* 2009) conclude by highlighting the fact that levels of many elements (including boron, iron, manganese, and vanadium) in the drinking water of Catania province often exceeded the maximum admissible concentrations and speculate a possible link. However, this study (Pellegriti *et al.,* 2009) did not measure levels of trace

elements in human samples and left open the question on trace elements being the real etiological agent in causing such increase.

The presence of endemic cretinism has long been reported in the North-Eastern Sicily (Squatrito *et al.,* 1981). Marked mental retardation was evident in all the affected subjects. In both myxedematous and neurological cretins, the urinary iodine excretion was very low, but not significantly different from that recorded in the euthyroid controls of the same area. Because of the proven presence of Iodine deficiency, the author suggested the immediate introduction of adequate iodine prophylaxis (Squatrito *et al.,* 1981). After 10 years another survey was carried out (Trimarchi *et al.*, 1990) indicating that in Sicily, endemic cretinism is a continuum of a variety of forms; among these the pure neurological and pure myxedematous forms represent the two extremes. The author (Trimarchi *et al.*, 1990) states the idea that endemic cretinism still represents a major public health problem in Sicily. The latest report found in international literature (Regalbuto *et al.*, 1998) is from 1998 and highlights the fact that the active iodine prophylaxis program carried out in the town of Troina in the years 1979-87 by iodinating the municipal water supply, caused a disappearance of goiter in schoolchildren in only five years. The author emphasizes the fact that the prevalence of goiter has decreased in all endemic areas probably because of the "silent prophylaxis", due to improved socio-economic conditions and industrial food consumption. However he notes that the persistence of endemic goiter confirms the inadequacy of the silent iodine prophylaxis and the need to immediately introduce an active iodine prophylaxis in Sicily.

A possible spatial-temporal cluster of multiple sclerosis (Nicoletti *et al.,* 2005, Nicoletti *et al.,* 2009) was reported in the small town of Linguaglossa. However, as yet no studies tried to investigate the relationship of this MS cluster with the volcanic environment. In order to explore this possible link, Chapter 6 describes an epidemiological case-control study carried out in Linguaglossa.

2.7 Multiple sclerosis

Multiple sclerosis (MS) is an autoimmune disorder resulting in inflammatory events, formation of sclerotic plaques and demyelinised lesions within the white matter of the central nervous system (see Figure 2-5 A). Between 2 and 2.5 million people suffer from MS around the world, of which the greatest part are young adults aged between 20 and 50. The peak of occurrence is at 30 years of age. Women are affected by MS twice as much as

men (Compston *et al.*, 2006c). There is a well known irregular geographic distribution of MS (Milo and Kahana, 2010) and ethnic differences in MS frequency (see Figure 2-5 B). Its prevalence varies between <5/100,000 people in tropical areas and Asia and >100–200/100,000 in temperate areas, especially in the large populations of Northern European origin, including United States, Canada, New Zealand and parts of Australia (Compston *et al.*, 2006a) (Milo and Kahana, 2010). Moreover MS frequency increases with latitude (Kurtzke, 2005) but hotspots are reported in South Mediterranean Europe such as Sardinia (prevalence 157/1,000,000) (Granieri *et al.*, 2000) and Sicily (Savettieri *et al.*, 2001).

MS is currently considered a multi-factorial disease and the autoimmune reaction causing myelin disruption is probably a result of the interaction between susceptible genes and loci, i.e. HLA allele DRB1*1501 (Dyment *et al.*, 1997), and one or more environmental triggers. However, a detailed knowledge of the pathogenesis and aetiology of MS is still missing. Several environmental factors have been hypothesized to be responsible for triggering MS as detailed in the introduction to Chapter 6. Exposure to trace elements is one of the factors considered to play a role. Table 2-2 summarises the main papers that analysed trace elements in tissues of MS patients.

Table 2-2: Summary of main papers reporting trace element imbalance in biological tissues of patients affected by MS

Element	Specimen	Variation	Reference
Mo	whole blood	↓	(Gellein *et al.*, 2008)
Cu, Zn			(Smith *et al.*, 1989)
Cu, Zn			(Rieder *et al.*, 1983)
Cu, I, Mn, S, Se, V	hair		(Ryan *et al*., 1978)
Co, Cu and Ni	blood	↑	(Forte *et al.*, 2005).
Be, Fe, Hg, Mg, Mo, Pb, Zn	blood	↓	(Forte *et al.*, 2005).
Ca, Fe, Sn, Zn	serum	↑	(Ristori *et al.*, 2011)

There is a variability in the clinical spectrum of MS (phenotypes) with various disease subtypes: relapsing remitting (RR), secondary progressive (SP), primary progressive (PP) and progressive relapsing (PR).

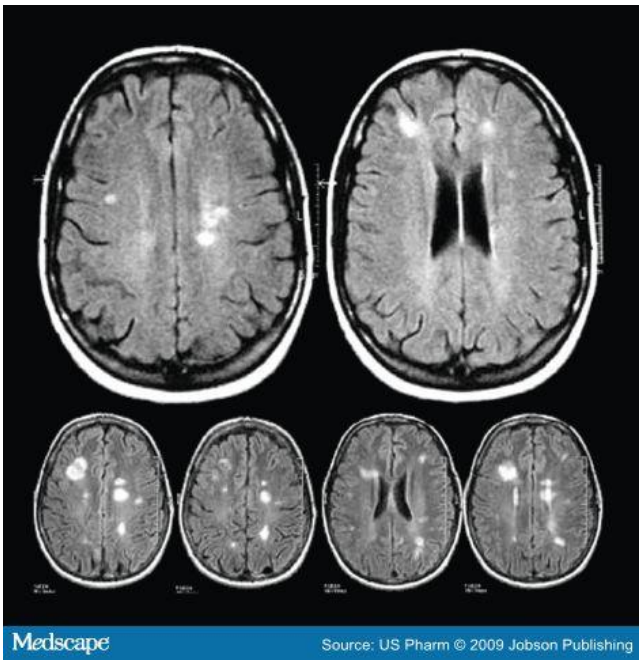
"RRMS is characterised by relapses (also known as exacerbations) during which new symptoms can appear and old ones resurface or worsen. The relapses are followed by

periods of remission, during which time the patient fully or partially recovers from the deficits acquired. During the relapse, in the relapsing-remitting MS acute stage, local inflammation and reversible demyelisation occur in the central nervous system (CNS). The SP form is dominated by irreversible changes such as permanent demyelisation and axonal loss. Approximately 50% of patients with RRMS convert to Secondary Progressive Multiple Sclerosis (SPMS) within 10 years of disease onset. PRMS is characterised by a steady progression of clinical neurological damage with superimposed relapses and remissions. PPMS is characterised by a gradual progression of the disease from its onset with no superimposed relapses and remissions at all" (quotation from Multiple Sclerosis Society, 2008).

MS treatment is currently based on symptoms relief with glatiramer acetate (a random polymer of glutamic acid, lysine, alanine and tyrosine) or with interferon beta, the latter has strongly modified the life quality of patients affected by MS.

In Chapter 6, an epidemiological case control study that was carried out on a Multiple Sclerosis (MS) cluster reported in Mt. Etna region and especially in the town of Linguaglossa. In this study, the roles played by trace elements, diet and life style as risk factors for MS were investigated.

a)



b)

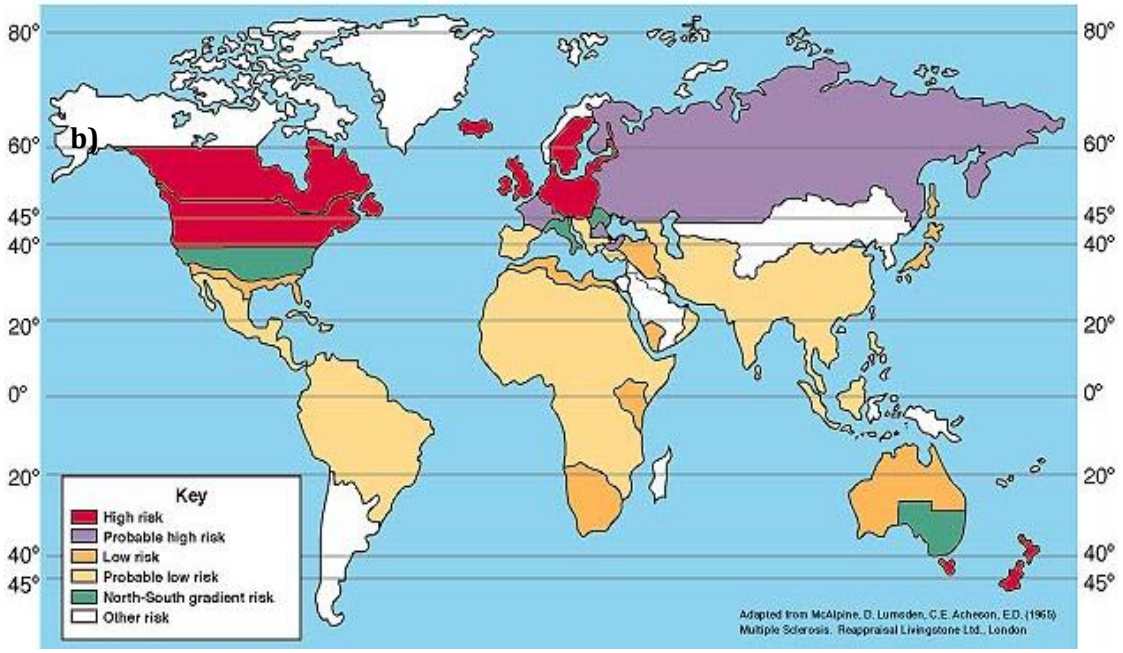


Figure 2-5: a) Magnetic Resonance (RMI) showing brain lesions (white parts) in a patient with multiple sclerosis. Source: Canadian Network of MS Clinics; b): world distribution of Multiple Sclerosis (source: www-medlib.med.utah.edu/kw/ms).

2.8 Trace elements in neurological diseases

Heavy metals and trace elements can be involved in the pathogenesis and/or aetiology of neurodegenerative and neurological diseases (Zatta *et al.*, 2003; Sayre *et al.*, 2000).

Some of the effects and correlation reported between heavy metals/trace elements and neurological conditions such as Alzheimer disease (AD), Multiple Sclerosis (MS), Amyotrophic Lateral Sclerosis (ASL) and Parkinson disease (PD) are briefly listed in Table 2-3.

Table 2-3 Reported neurological effects of trace elements and heavy metals

Element	Neurological effect/alteration	Reference
As	Effects on Learning, memory, concentration, decrease in verbal IQ and hearing alteration	(Bencko and Symon, 1977)
Ni	Increased in blood of Multiple Sclerosis patients	(Forte *et al.*, 2004)
Co	Increased in blood of Multiple Sclerosis patients	(Forte *et al.*, 2004)
Hg	Minamata disease	
Zn	Alzheimer Disease (found in senile plaques)	(Lovell *et al.*, 1998)
Cu	Alzheimer Disease (found in senile plaques) Increased in blood of Multiple Sclerosis patients	(Lovell *et al.*, 1998, Forte *et al.*, 2004)
Pb	Motor neuropathy	(Fluri *et al.*, 2007)
Mn	Manganism – Parkinson like disease	(Dobson *et al.*, 2004)
Fe	Alzheimer Disease (found in senile plaques)	(Lovell *et al.*, 1998)
Se	Exposure to inorganic Se increase risk of Amyotrophic Lateral Sclerosis	(Vinceti *et al.*, 2010)

To cite just some of the known effects of metals and metalloids on CSF, acute occupational exposure to manganese (Mn) is involved in the generation of a Parkinson-like neurological syndrome known as Manganism, while Mn chronic exposure from drinking water has an impact on children's intellectual function in Araihazar, Bangladesh (Wasserman *et al.*, 2006). Copper, zinc and iron are found in the senile plaques typical of Alzheimer disease and are known to modulate the aggregation state and toxicity of the beta amyloid *in vivo* (Lovell *et al.*, 1998) and derived peptides *in vitro* (Giuffrida *et al.*, 2007).

This thesis presents two different studies carried out on patients affected by neurological conditions, as detailed below:

In Chapter 6 a bio-monitoring study on trace elements in urine and toenails of MS patients and controls from Linguaglossa was carried out; in Chapter 7, a bio-monitoring study of

CSF for trace element composition of MS and controls was carried out. Experimental levels are compared to relevant literature.

In both cases, the trace element composition of the biofluids and biotissues can be used as biomarkers of past/recent exposure as a snap-shot in the history of exposure for each volunteer, or as a consequence of an alteration of the metals/trace elements balance in body compartments as result of the pathology.

3. Biological samples for human biomonitoring

3.1 The multidisciplinary methodology

In the present study a variety of techniques was applied for human biomonitoring with different purpose, as detailed in the Chapter 1 and depicted in Figure 2-6.

Individual information on food consumption has been collected by means of a questionnaire that also included questions on several variables that could act as confounding factors. Metallomics (by means of ICP-MS and HPLC-ICP-MS) were applied to define the trace element profile of urine and other biological samples and Metabolomics (by mean of NMR spectroscopy) was applied to look for differences in the urinary metabolomic profile in relation to arsenic and selenium exposure and other factors.

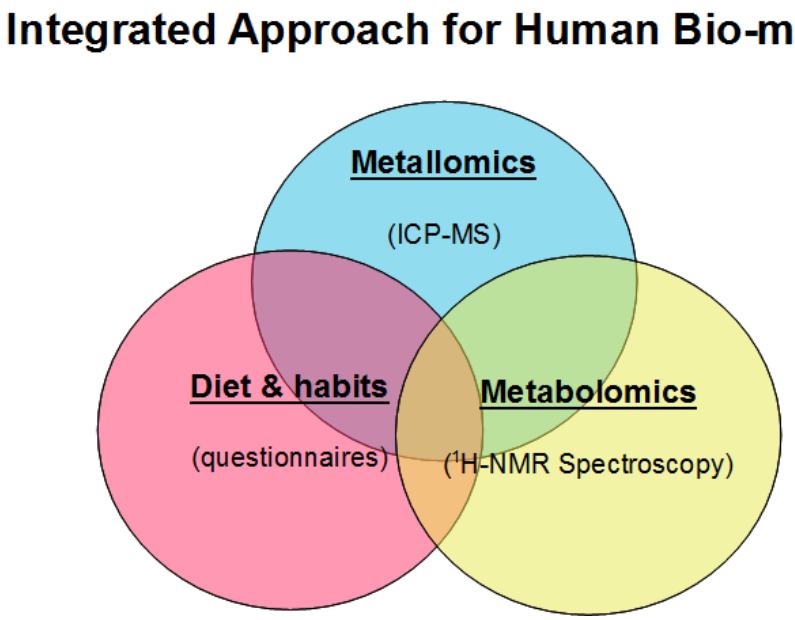


Figure 2-6: Multidisciplinary approach for human biomonitoring applied in this study

3.2 Ethics

The physical, emotional and psychological safety of the human volunteers participating in this study has been guaranteed. Before collection of any sample or personal data, a research plan has been developed and approved by the Faculty of Health and Life Sciences Human Research Ethics Committee of De Montfort University. In addition, when collaborative projects were in place with other institutions, a local ethical committee provided approval for the study and the sampling methodology (namely the University of Catania, Italy and the University of Chicago, USA).

For sampling carried out from De Montfort University, each volunteer involved was given: a presentation letter, a questionnaire, an informed consent form to be signed, a labelled plastic bottle for urine collection, separate plastic bags for nails and hair collection. Questionnaire, bottles and plastic bags were labelled with unique individual alphanumeric code.

The letter given to the volunteers contained:

- Research purpose
- A description of the procedure to be followed for sampling
- A statement of voluntary participation
- The benefit to the subject and to others
- Description of procedure to ensure data protection/confidentiality/privacy
- The possibility to withdraw at any moment
- An explanation of what will happen to the data at the end of the research period
- Information about what will happen with the results at the end of the research

Volunteers signed an informed consent according to terms set by the Declaration of Helsinki (World Medical Association, 2000) and the Clinical Trial Directive 2001/20/EC (European Parliament, 2001).

Data sampling and storage followed the EU rules on data protection and storage Directive 95/46/EC). Specifically: i) a hard copy of the data was stored in a secure locked cabinet ii) the electronic copy of the data was managed by the researcher and delegated persons, with restricted access by a password; iii) multiple copies (with restricted access) of the electronic data were created including a copy on an external hard disk, stored in a different location from the hard copy.

3.3 Questionnaires

In order to acquire data on the studied populations, a self administered questionnaire was developed.

The questionnaire included a two day food diary organised in breakfast, lunch, dinner and other and was completed by the volunteers. The first section of the questionnaire was dedicated to demographics, intake of medications, smoking and ethnicity. A detailed section was dedicated to water intake, including quantity and the type of water (bottled, tap, well and fountain).

A Food Frequency Questionnaire (FFQ) was used. In this study the consumption of 36 key foods was investigated, with specific attention on staple food (pasta, bread and rice) and fish and different types of fruits.

For pasta, rice, bread and fish two close questions were formulated; one on the portion (in grams or slices according to the type of food) and the other on the frequency (7 categories from 'rarely' up to '2 times per day'). The final amount was calculated multiplying the portion for the frequency and then scaling to one day unit. For pasta, rice and bread, separate categories were used for white and brown. An open question on the type of pasta or rice was added.

For other foods, only the frequency of intake was investigated in 8 categories from 'never' up to '2 times per day'. Specific questions were dedicated to the consumption of organic and locally grown food.

Information on occupation and other additive possible sources of exposure to trace elements were detected as well. The history of residence was also recorded.

3.4 Urine

Urine is formed in the morphological and functional unit of the kidney, the nephron, from filtration of dissolved substances in the blood that need to be excreted from the body.

Urine is the most frequently used matrix in humans to quantify environmental or occupational exposure to pollutants, especially for substances with short biological half-lives (Castano Esteban, 2009).

Because of its ease of sampling, urine is an extremely valuable biofluid for human bio-monitoring and it has been widely applied to assess human exposure to arsenic and other trace elements in both European (Hamilton *et al.*, 1994a) and non-EU context (Agusa *et al.*, 2009).

Spot urine collection is the most widespread method for urine collection for reason of time, cost and simplicity. The major disadvantage in spot urine samples, compared to 24 hour collection, is the variability in the volume of urine and concentrations of endogenous and exogenous chemicals from void to void (Barr *et al.*, 2005). Different approaches can be used to minimize the variations in urinary arsenic, trace elements and metabolites. The most widely used approach are specific gravity (Nermell *et al.*, 2008b), and creatinine normalization (Kavanagh *et al.*, 1998; Iversen *et al.*, 1998).

3.5 Sampling and storage

Urine samples were collected in plastic bottles (HDPE or PP), avoiding glass. Bottles were soaked with 5% nitric acid and then washed with milliQ water. Nail samples were collected in transparent plastic bags which were kept closed until the day of the analysis.

After sampling, the collected urine samples were frozen within few hours. Shipment of samples to the UK involved packaging them in dry ice and following standard procedures for the shipment of category B biosamples. After defrosting once for measuring specific gravity and performing a Combur test, samples were aliquoted and kept frozen till the day of the analysis.

Cerebrospinal fluid samples were collected in transparent plastic tubes.

3.6 Creatinine or Specific gravity?

Creatinine is a waste product of the dehydration of creatine and creatine phosphate in muscle metabolism. The greatest part of creatine is accumulated in skeletal muscle. Approximately 2% of body creatine is converted into creatinine every 24 hours (Barbanel *et al.*, 2002). Several studies measuring arsenic levels in urine have used creatinine normalization (Kavanagh *et al.*, 1998, Iversen *et al.*, 1998). However, some criticisms have been directed at this approach since the rate of excretion can vary greatly between subjects as a function of factors such as body weight and fat free mass (Baxmann *et al.*, 2008) and dietary protein intake (Mayersohn *et al.*, 1983). Nermell *et al*. (Nermell *et al.*,

2008b) concluded that specific gravity may also be influenced by age, gender, and body size, but the influence is less than for creatinine. Multiple regression analysis of population groups (age, sex, racial groups) has been proposed to allow the analyte concentration to be independent from creatinine variations (Barr *et al*., 2005).

Nermell *et al.* (Nermell *et al.*, 2008b) found urinary creatinine levels were significantly associated with urinary arsenic from the analysis of 1466 samples of rural Bangladesh population and preferred specific gravity (SG) for the adjustment of variations in urinary dilution.

3.7 Measurement of Specific Gravity

The current study used specific gravity to correct for different filtration rates. SG of urine samples was measured using a digital portable refractometer from ATAGO (Figure 2-7 A). This device allows the measurement of the specific gravity from a volume as little as 300 µl in a few seconds per sample. Measurement is performed indirectly by detecting the angle of light refraction between air and the urine sample (Figure 2-7 B). The refraction increases with the total solute concentration in urine. The normal range of SG is 1.001-1.020 (Barbanel *et. al*, 2002).

3.8 Combur test

Quick and cheap analysis of urine samples can be performed by mean of strip test (Combur test by Roche in Figure 2-8). This test allows the screening of a number of clinical parameters detailed in Figure 2-8. The reagent paper and underlying absorbent paper are held in place on a stiff white carrier foil with a fine porous nylon mesh laminated to the foil itself. The analysis is done by quickly dipping (less than 1 second) the strip into the urine and performing a visual or instrumental reading. A Combur test allows the identification of slight pathological changes in the urine (see Figure 2-8).

3.9 Trace element levels in urine

Urine is a good biomarker of recent exposure to trace elements. The meaning of some biomarkers in urine is shown in Table 2-4 in relation to half-life and time of exposure.

Table 2-5 shows different trace elements baseline levels reported for urine in Europe and abroad. Data are relative to general population in areas where a main geogenic or

anthropogenic pollution were not reported. In the general population the main factors affecting the urinary composition are likely to be soil composition, diet, groundwater and personal habits such as smoking. Reference values set for different groups are also reported in Table 5-2. It should be noted that not all the studies used a great number of volunteers, some studies are quite dated and background pollution levels might have changed (i.e. for nickel); another limitation of this type of comparison is that each group of research publishes data based on different normalization methods (here data are reported without any normalisation) and using different descriptive statistics.

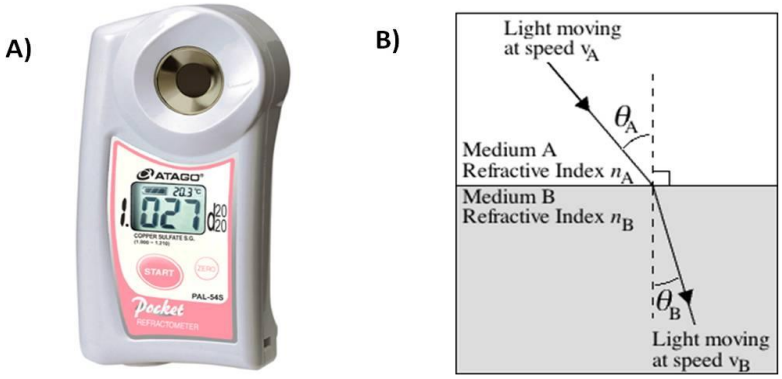


Figure 2-7: A) Digital refractometer used to determine urine specific gravity; B) the change of refraction angle of light passing from one medium into another with different refractive index source: www2.ups.edu/faculty/hanson/labtechniques/refractometry/theory.htm

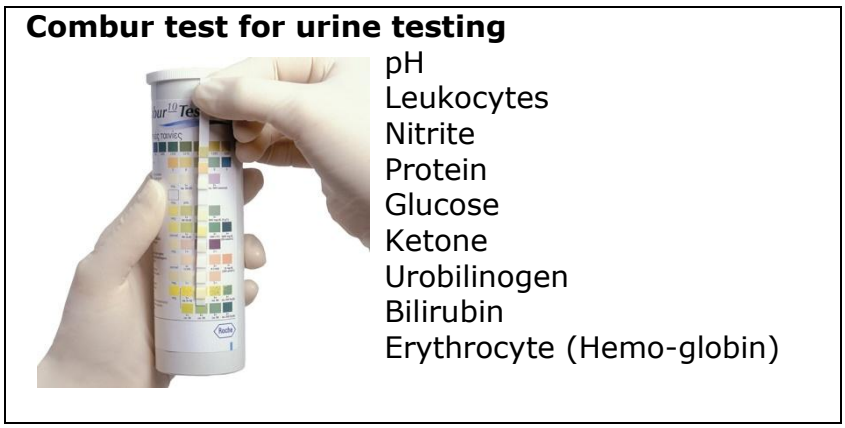


Figure 2-8: Combur test (Roche) used to screen main parameters in urine

It is noteworthy that total arsenic accounts for different species present in urine and is not *per se* a sufficient estimation for risk assessment.

Table 2-4: The meaning of some biomarkers in urine and serum from (Alimonti *et al.*, 2010)

Element	Half-life	Matrix	Type of exposure
Aluminium	8-14 h 42 days	urine serum	recent chronic
Arsenic	2-4 days	urine	recent
Cadmium	7 h 1-3 months	urine blood	recent
Cobalt	24 h	urine	recent
Chromium	2-3 days 25-35 days	urine blood	recent
Mercury	1-3 months	urine	recent-chronic
Lead	1-2 months	blood	recent

3.10 Arsenic species in human urine

Human urine is a complex mixture of inorganic and organic arsenic species. The comparative study of arsenic metabolites in urine and unchanged species give an insight into metabolic processes in a population or subgroups (Brima *et al.*, 2006c). Once ingested, arsenic is involved in human metabolic processes, and the excretion of arsenic species is the result of both exposure and biotransformation. A schematic representation of this is in Figure2-9.

The main arsenicals in humans urine are DMA(V) and AB (Brima *et al.*, 2006b). The proportion of the arsenic species in urine is typically 60–80% DMA, 10–20% MMA, and 10–20% inorganic arsenic (Le *et al.*, 1994; Buchet *et al.*, 1981) in individuals who do not eat food of marine origin, such as fish, shellfish, and algae.

Table 2-5: Reference values of certain trace elements reported for urine in different countries [µg/L] adapted from (Batista *et al.*, 2009)

Country	Parameter	n	Cu	Se	Co	Zn	Mn	Ref.
USA	25th-95th	500	/	/		/	< 0.2- 3.33	1
UK	median	> 100	11.7	16.2		/	0.3	2
UK	ref value	180	4.7-29.3	6.0-43.3		/	0.09- 1.89	3
GERMANY	min-max	14	/	/		/	/	4 - 5
GERMANY	geomean (60th-95th)	87	8 (9-13)	12 (14-24)	0.18 (0.16- 1.53)	207 (265 -692)	0.063 (0.079 0.21)	6
FRANCE	5th-95th	100	4.3-12.1	10.5-58.1	0.16-1.14			7
SWEDEN	min-max	19	1.9-15.9		0.06-0.51			8
ITALY	ref value		4.20-50	2.1-30.9		266-846		9
BRAZIL	5th-95th	412	2.2-18.4	5.2-12.4		/	0.5-4.4	10

Country	Parameter	n	Pb	As	Cd	Cr	Ni	Ref.
USA	25th-95th	500		/			/	1
UK	median	> 100		3.65			0.84	2
UK	ref value	180		0.7-19.4			0.25-5.0	3
GERMANY	min-max	14		/			0.6-3.4	4 - 5
GERMANY	Geomean (60th-95th)	87	0.6 (0.7 -2.1)	13 (14-143)	0.17 (0.18 -0.42)	0.127 (0.15 -0.37)	0.30 (0.54 2.5)	6
FRANCE	5th-95th	100	0.01-2.14	/	0.06-0.48	/	0.49-4.06	7
SWEDEN	min-max	19	0.3-2.0	/	0.044-0.358	/	0.27 - 3.68	8
ITALY	ref value	…		2.3-31.1				9
BRAZIL	5th-95th	412		/			0.1-4.2	10

1 = (Paschal et al., 1998); 2 = (White and Sabbioni, 1998); 3= (White and Sabbioni, 1998); 4 = (Wilhelm *et al.*, 2004); 5=(Schramel *et al.*, 1997)
6 =(Heitland and Köster, 2006); 7 = (Goullé *et al.*, 2005); 8 = (Rodushkin and Ödman, 2001); 9= (Minoia *et al.*, 1990); 10 = (Batista *et al.*, 2009).

Other reported species are As (III) and As(V). MA(V) is generally low in urine but it is increased under certain conditions such as alcohol consumption. DMA(III) and MA(III) are highly reactive and unstable compounds that can be found in urine by means of sophisticated analytical approaches (Francesconi and Kuehnelt, 2004). For instance DMA(III) in urine is converted to DMA (V) in 17 hours even at -20°C. Cationic compounds in urine such us TMAO, TETRA and AC are present at very low levels (Francesconi and Kuehnelt, 2004).

Furthermore, ingestions of arsenosugars through fish induces formation of other human urinary arsenicals including oxo-dimethylarsenoethanol (oxo-DMAE), trimethylarsine oxide, oxo-dimethylarsenoacetate (oxo-DMAA), thio-dimethlyarsenoacetate (thio-DMAA), thio-dimethylarsenoethanol (thio-DMAE), and thio-arsenosugar (Raml *et al.*, 2005).

Ingestion of arsenolipids leads mainly to the formation of DMA and other soluble arsenicals excreted in urine. However, a small proportion of arsenolipids are excreted, such as oxo-dimethylarsenopropanoic acid, thio-dimethylarsenopropanoic acid, oxo-dimethylarsenobutanoic acid, and thio-dimethylarsenobutanoic acid (Schmeisser *et al.*, 2006).

3.11 Hair and nails

Both hair and nails are non-invasively collected matrices for human biomonitoring and their use is promoted as an ethically appropriate, cost-efficient and toxicologically relevant alternative for many biomarkers that are currently determined in invasively collected matrices (Smolders *et al.*, 2009). Both hair and nails are rich in keratin, which is a structural protein with a high content of sulphur.

Hair is essentially composed of cylindrical structures made up of tightly compacted cells growing from a small organ called follicle (see Figure 2-10). The diameter of a human hair ranges from 15 – 150 μm. Human hair is made of 65-95% of proteins, 15-35% of water and 1-9% of lipids (Harley, 1993). The same chemical structure is shared with nails, that are however richer in structural proteins. Both hair and nails, because of their keratin content, strongly bind trace elements circulating in the body capturing them from the blood stream (Hopps, 1977). Hence they represent a good biomarker of past exposure to

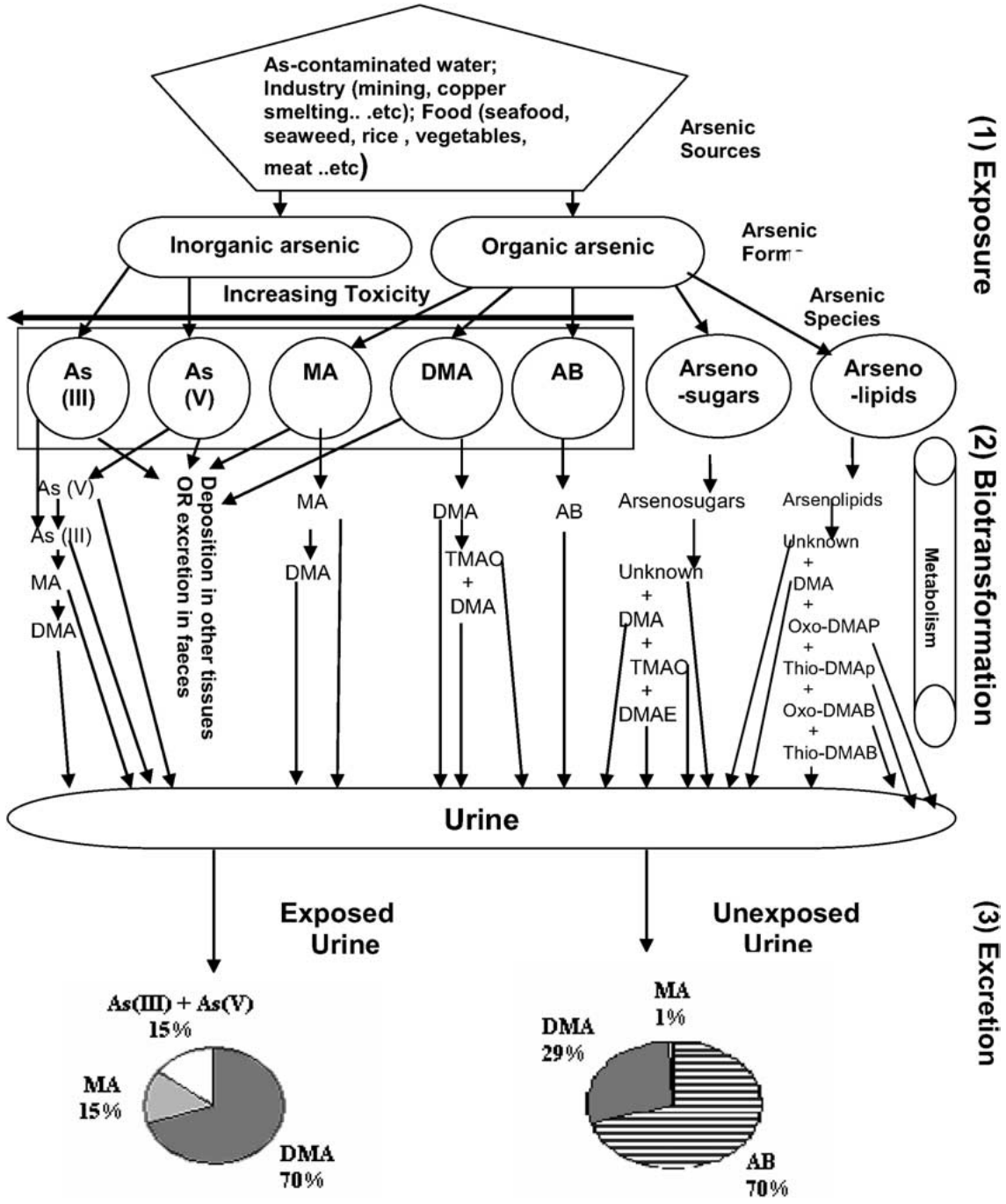


Figure 2-9: A simplified flow chart describing the fate of As in humans starting from exposure and ending with excretion of As in human urine for exposed and unexposed populations. The Figure illustrates the current understanding of As metabolism in humans based on previous studies reported in the literature. This figure is from (Brima *et al.*, 2006c)

trace elements. Hair and nails have been successfully used to measure both internal and external exposure to a wide variety of organic and inorganic pollutants and especially trace elements. Hair (Cleland *et al.*, 2009; Brima *et al.*, 2006b; Iversen *et al.*, 2003) and

finger/toenails (Kile *et al.*, 2005; Button *et al.*, 2009) are used alone or in conjunction with urine for monitoring arsenic (and other trace elements) exposure. There are several advantages in using toenails as a biomonitoring tool: 1) toenail clippings are non invasive; 2) it allows for easy storage because of stability; 3) as opposed to hair or fingernail samples, the likelihood of external contamination is lower (Hopps, 1977); 4) toenails reflect exposure occurring 6-12 months before sampling while urine generally reflects exposure in the last few days, according to element half life (Heitland and Köster, 2004, Laohaudomchok *et al.*, 2011); and 5) arsenic tends to accumulate in higher concentrations in toenails than in fingernails. Garalnd *et al.* (Garland *et al.*, 1993) showed that levels of trace elements in nails are significantly correlated over time, and one-time measurement might be reflective of long-term exposure. There is a linear correlation between arsenic in drinking water and hair/nails content (Karagas *et al.*, 2000), (Schmitt *et al.*, 2005), as shown in Figure 2-11 which shows a positive relationship between arsenic in drinking water and hair arsenic content on a small population from Croatia.

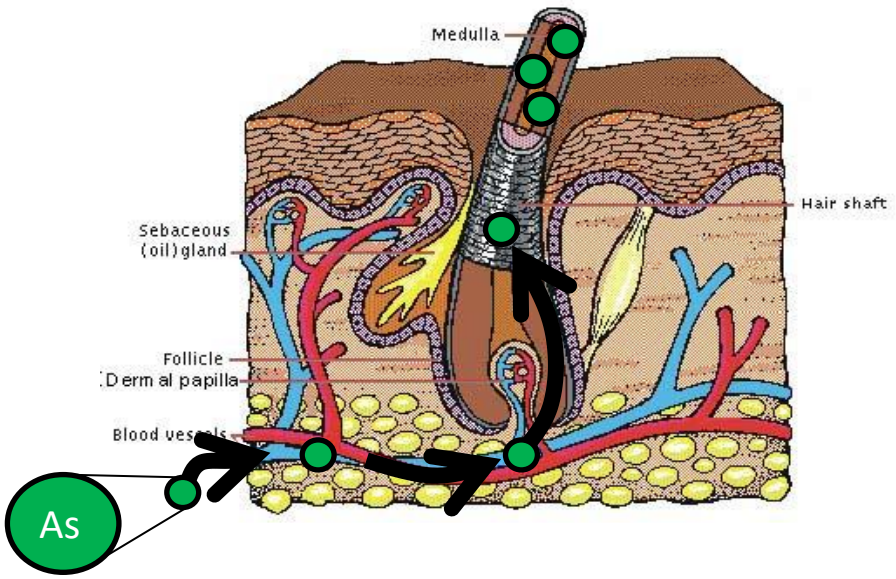


Figure 2-10: Arsenic and other trace elements pass through the blood vessels into the bulb becoming part of the cyto-chemical structure of hair, modified from http://www.dolcera.com/wiki/images/Hairbasics.jpg

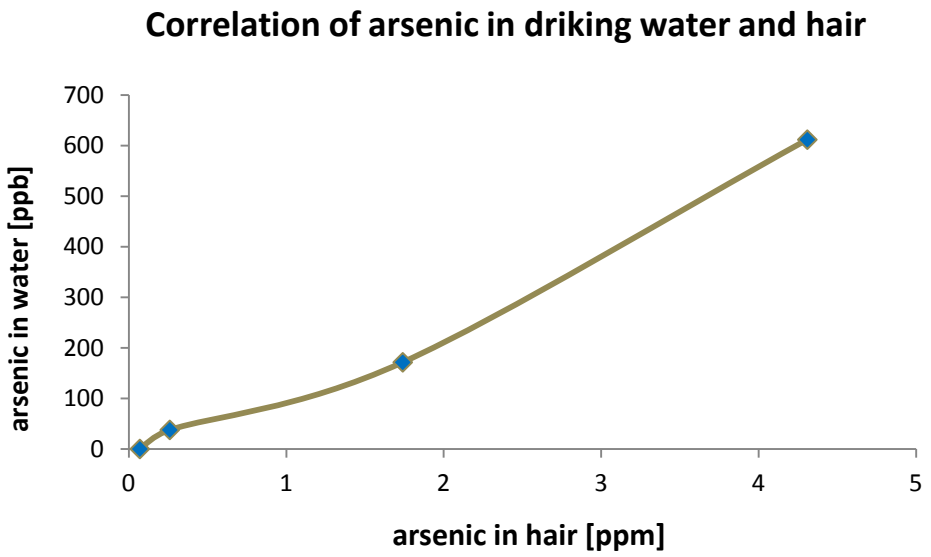


Figure 2-11: Relationship between arsenic content in drinking water and hair from a local population in Croatia (n=62); points are based on average. Data reported by (C ´ avara *et al.*, 2005).

Amongst the disadvantages of using hair and nails for monitoring exposure to trace elements is the difficulty in differentiating between internal and external sources of contaminants and the widespread use of hair treatment products (ATSDR, 2001, Zhang *et al.*, 2007). Diverse decontamination procedures are adopted in different studies including acetone and sonication (Slotnick *et al.*, 2005), acetone and water (Slotnick *et al.*, 2007), 1% Triton X-100 solution (Kile *et al.*, 2005) or multistep washing (Dean A. Bass *et al.*, 2001). The latter approach was adopted in the current study (Chapter 6) to decontaminate toenails from the Mt. Etna study group before preparation for trace element analysis.

Some values from exposed and non-exposed populations are reported in Figure 2-12 that is based on Table 2-6 for both finger and toenails. It is noticeable that even if the UK is generally not affected by arsenic, an exceptionally high level of arsenic in toenails of a population from the UK residing in Devon has been reported. This population resides in an area close to a former tin mine (Button *et al.*, 2009). In addition, Table 2-7 presents data for other trace element levels in toenails reported for an Italian population from Emilia Romagna by (Bergomi *et al.*, 2002).

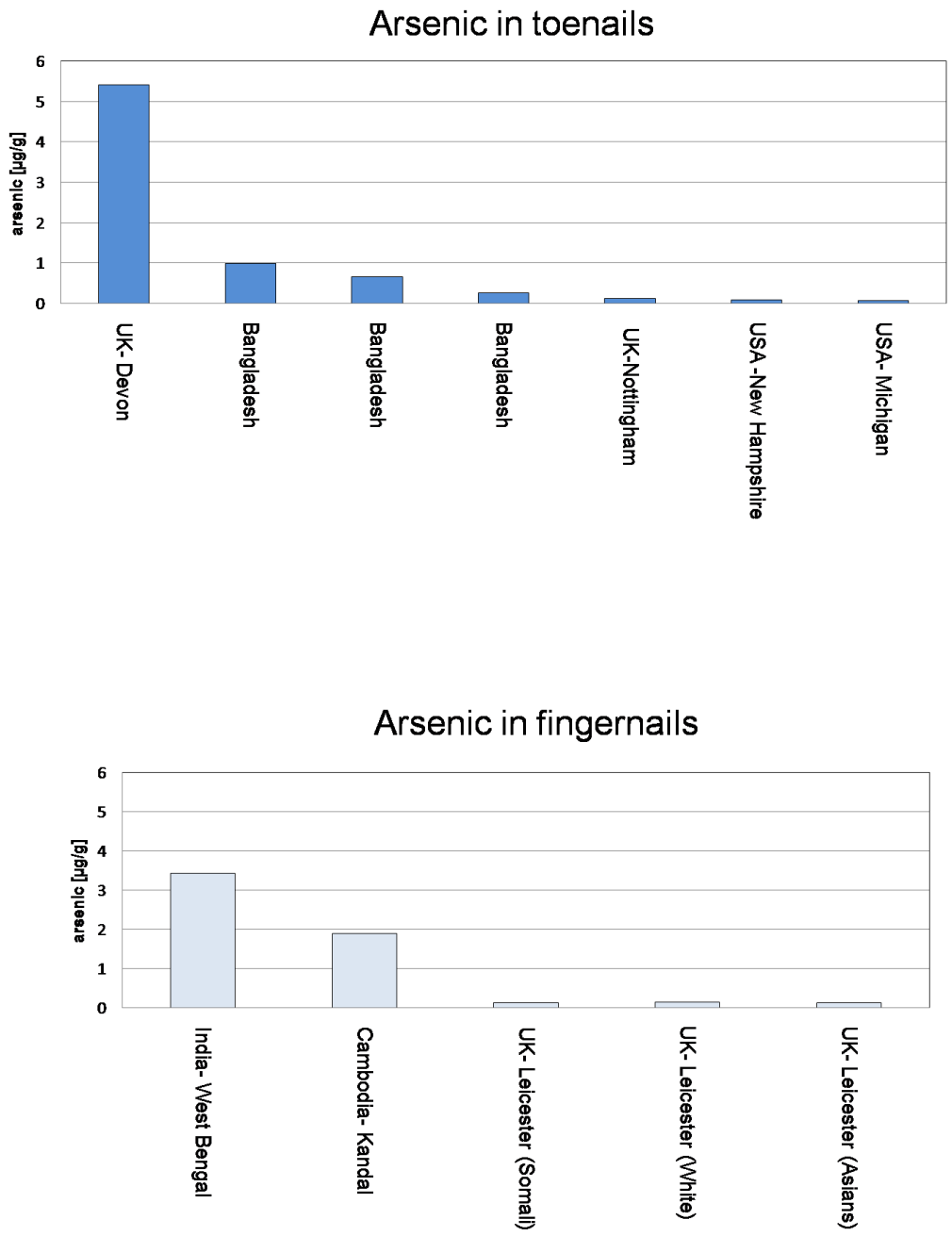


Figure 2-12: Arsenic in nails of different study populations, references as in Table 2-6.

Table 2-6: arsenic content in fingernails and toenails from people living in different parts of the world

fingernails*							
Country - Region (ethnicity)	**n**	**Min***	**Max***	**Mean***	**Type^**	**As in drinking water'**	**Reference**
India- West Bengal	44	0.17	14.39	3.43	g	>50 (100-230)	(Samanta *et al.*, 2004)
Cambodia- Kandal	40	0.1	7.95	1.9	a	153 (0.21-943)	(Gault *et al.*, 2008)
UK- Leicester (Somali)	22	NR	NR	0.116	a	<10	(Brima *et al.*, 2006b)
UK- Leicester (White)	20	NR	NR	0.141	a	<10	(Brima *et al.*, 2006b)
UK- Leicester (Asians)	21	NR	NR	0.117	a	<10	(Brima *et al.*, 2006b)

toenails*						
Country - Region	**n**	**Min***	**Max***	**Mean***	**As in drinking water'**	**Reference**
UK- Devon	8	0.858	25.981	5.406	<10, soil exposure	(Button *et al.*, 2009)
Bangladesh	100			0.988		(Kile *et al.*, 2005)
Bangladesh	100	340	4.87	0.66	42.4 (ND-138)	(Kile et al., 2005)
Bangladesh	100	0.0552		0.267	55.2 (ND-752)	(Kile et al., 2005)
UK-Nottingham	9	0.073	0.273	0.122	<10	(Button *et al.*, 2009)
USA -New Hampshire	506	<0.01	0.81	0.09	0.29 (0.002-66.6)	(Karagas *et al.*, 2000)
USA- Michigan	488	0.003	1.26	0.07	0.55	(Slotnick *et al.*, 2008)

(*) [µg/g]; (') [µg/L]; (^) arithmetic mean (a) and geometric mean (g).

Table 2-7: Trace elements levels toenails of an healthy non occupationally exposed group from Italy *

Element	n	median	25th	75th
Cd	40	0.05	0.05	0.05
Se	40	0.59	0.53	0.7
Mn	40	0.8	0.42	1.72
Pb	40	1.22	/	/
Cr	40	1.23	0.58	3.14
Cu	40	4.24	3.57	6.48
Co	40	18	9	41
Fe	40	22	16	72
Al	40	37.5	22.0	87.0
Zn	40	102	92	123

(*) values in [µg/g] from Bergomi *et al.,* 2002

3.12 Cerebrospinal fluid

The cerebrospinal fluid (CSF) is a dynamic, metabolically active transparent fluid that occupies the subarachnoid space and the ventricular system around and inside the brain and spinal cord. CSF has several important functions for the brain including: a) shock absorption, b) nutrition for neutrons and glial cells c) removal of catabolises, d) chemical communication since CSF contains cytokines, hormones, metabolites and neurotransmitters.

CSF can be obtained via lumbar puncture (see Figure 2-13) and its biochemical analysis is used in diagnostics for the evaluation of inflammatory conditions, infections of the brain, spinal cord and meningitis and for other conditions. Table 2-8 shows typical concentrations of some biochemical parameters screened in CSF in normal and neurological conditions. Total proteins (and albumin) in CSF is used as an indication of the blood-brain barrier (BBB) integrity. The BBB is a physical structure aimed to filter macromolecules and biohazards to prevent them from reaching the CSF. An increase in protein content in CSF is an indication of leakage, and the albumin CSF/serum concentration quotient (Qalb) can also be used to evaluate blood-brain barrier integrity and used in conjunction to other criteria. The single most informative analysis is a qualitative assessment of CSF for IgG in the diagnosis of multiple sclerosis (Freedman *et al.*, 2005).

Table 2-8: typical concentration for CSF parameters in normal and some neurological conditions

	Total proteins	Glucose ratio	Lactate	Cell count	Typical cytology
Normal	< 0.45	>0.4-0.5	< 1.0-2.9	<15	MNC
Acute bacterical meningitis	↑	↓	↑	>1000	PNC
Viral neuron-infections (meningo/encephalitis)	=/↑	=/↓	=	10-1000	PNC/MNC
Autoimmune polyneuropathy	↑	=	=	=	
Infectious polyneuropathy	↑	=	=	↑	MNC
Subarachnoidal haemorrhage	↑	=	=	↑	ER, MC, SI, MNC
Multiple Sclerosis	=	=	=	=/↑	MNC
Leptomeningeal metastases	↑	=/↓	NA	=/↑	Malignant cells, mononuclears

MNC: mononuclear cells, PNC: polymorphonuclear cells, ↑: increased, ↓: decreased, =: within normal limit, ER: erythrocytes, MC: macrophages, SI: siderophages, NA: evidence not available. (Deisenhammer *et al.*, 2011)

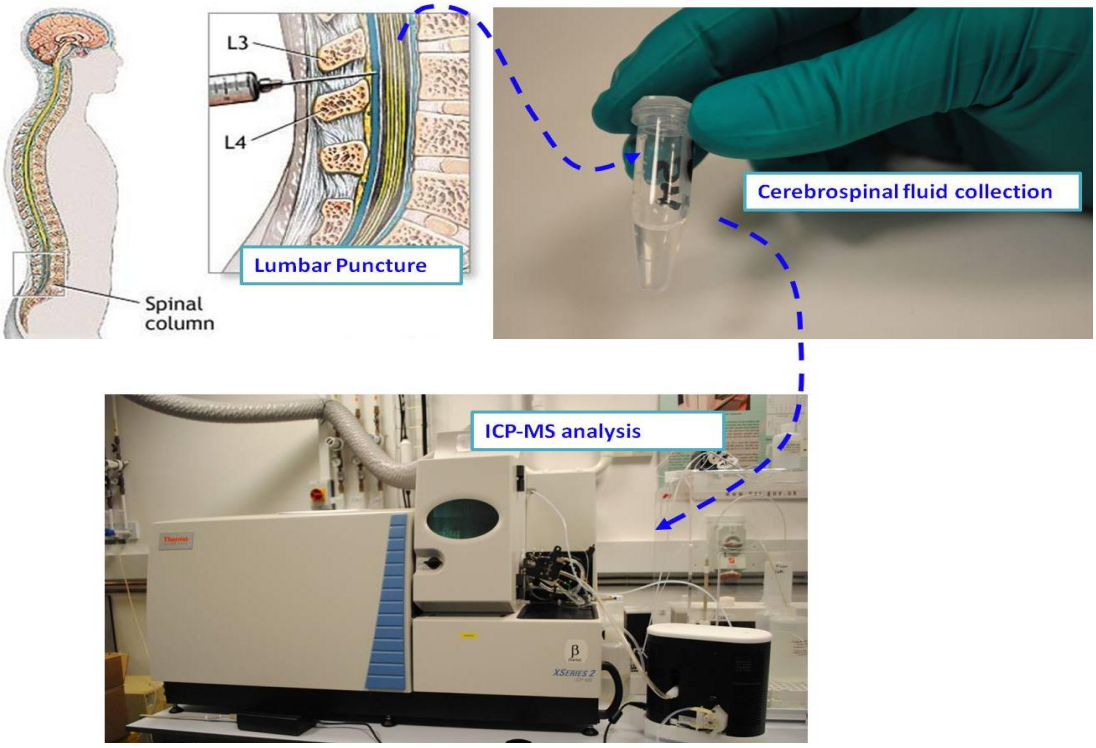


Figure 2-13: spinal column and lumbar puncture to obtain CSF in order to perform ICP-MS analysis. First picture from http://reidhosp.adam.com

Because of the very invasive sampling procedure, CSF is not used as a routine biomarker of trace elements exposure, however trace element patterns have been screened in patients affected by neurological diseases and certain imbalances have been found in relation to conditions such as Parkinson's (Alimonti *et al.*, 2007a) and Alzheimer's (Gerhardsson *et al.*, 2008) disease.

Levels of some trace elements reported for CSF in the literature are summarised in Chapter 7 and compared to the baseline levels found in MS patients.

It is important to highlight the fact that, in general, these studies have the drawback of the lack of suitable control population. The procedure to collect CSF is invasive and it is not ethically acceptable to collect CSF from healthy controls. Therefore, the greatest part of the studies use as controls a non affected population from the considered condition, but with some kind of pathology, with all the limitations that this approach produces. Chapter 7 of this thesis report a comparative study on CSF of patients affected by MS and controls affected by migraines carried out in order to investigate differences in trace elemental composition of CSF related to pathology and its progression.

4. Determination arsenic and other trace elements in biological samples

A range of techniques can be used to estimate the content of arsenic and other trace elements in urine and other biological tissues. To deal with the concentrations as low as ppb and sometimes parts per trillion (ppt) found in biofluids, advanced analytical techniques are required. Applied approaches include Hydride Generation Atomic Absorption Spectrometry (HG-AAS)(Wen *et al.,* 2011), Graphite Furnace Atomic Absorption Spectrometry (GF-AAS)(Delgado *et al*., 2011), Inductively coupled plasma-atomic emission spectrometry (ICP-AES)(Słojewski *et al.,* 2010), hydride generation atomic fluorescence spectrometry (HG-AFS) (Ito *et al.*, 2010), Inductively coupled plasma-mass spectrometry (ICP-MS) (Newcombe *et al*., 2010) and X-ray methods such as X-ray fluorescence (XRF)(Kempson *et al*., 2009), electrochemical techniques including potentiometry and voltametry and Synchrotron-based techniques including X-ray Absorption Near Edge Structure (XANES) and Extended X-Ray Absorption Fine Structure (EXAFS) and Neutron Activation Analysis (NAA)(Brockman and Schell, 2011, Pazirandeh *et al.*, 1998).

4.1 ICP-MS

ICP-MS (Figure 2-14 and 2-15) is a technique that allows the detection of metal (and metalloids) concentration in the range from ppm to sub-ppb. It is a versatile technique with a low detection level and high sensitivity. It offers a wide linear dynamic range, high throughput, multi-element capability, relatively simple spectra and the possibility to conduct isotopic analysis (Beauchemin, 2002).

The different processes occurring in an ICP-MS analysis are shown Figure 2-14 along with the representation of the structure of an ICP-MS. Pictures in Figure 2-15 shows the Agilent 7500 ICP-MS used at the Aberdeen University and applied in Chapters 3 and 4 of this study along with cones and torch.

In the sample introduction the liquid sample (diluted or digested) is sucked using a peristaltic pump into a nebuliser where it is converted into an aerosol by mean of a spray chamber. The sample is then transported into the plasma torch after a selection of the smaller droplets. During this process the larger droplets are rejected and the smaller ones, which will be efficiently ionised, reach the centre of the plasma.

During the ionization the small droplets reach the torch with a carrier gas that is usually argon; the torch is made of concentric quartz tubes (Figures 2-14 C and 2-15 E). The sample and the carrier gas pass across the inner tube while in the external concentric tubes, argon is used as cooling gas. A radiofrequency (RF) generator produces an oscillating current in an induction copper coil that wraps the tubes. The induction coil creates an oscillating magnetic field. The magnetic field in turn sets up an oscillating current in the ions and electrons of the support gas. The plasma is generated in the stream of argon contained in the torch. The intense magnetic field created by the electric current causes collisions between free electrons and Ar atoms, producing ions and more electrons, until a stable, high temperature plasma is formed.

In this way, the plasma torch is responsible for sample ionization: firstly the sample droplet approaches the plasma, then it is atomised and finally ionised. The argon plasma can reach very high temperatures (up to 10,000 K) and produces a very efficient ionization.

The aim of the plasma is to form positively charged ions from the sample aerosol, with a good efficiency. To ensure good results from samples with varying matrices, plasma loading should be optimized to maintain high ionization temperatures while retaining good sensitivity. The goal is to achieve a degree of matrix decomposition and analyte ionization as high as possible.

The equation that regulates the ionization in the plasma is the Saha equation:

$$a/1\text{-}a = [(2p\ m\ k\ T)^{3/2} / N_e\ h^3] * [2\ Z/Z] * e^{-v/kT} \quad \text{(Equation 3)}$$

a = Ionization efficiency
N_e= Electronic density
m = Ion mass
V= Ionization potential for the element
K = Boldzman constant
T= Temperature of the gas
h = Plank's constant
Z = Partition function

The formation of ions from the sample atoms is achieved by the removal of a single electron. This occurs with varying ease and efficiency for different elements. This variation is usually quoted as the "Ionization Efficiency" for each element, which is a function of the first ionization potential of the element together with estimated values for plasma electron temperature and density.

The positively charged ions that are produced in the plasma are extracted into the vacuum system via a pair of interface "cones" (Figure 2-15 B and C). The cones are essentially metal plates with central orifices through which the ions pass. Small orifices are used, typically 1 mm diameter or less, to maintain the high vacuum in the mass spectrometer region. Cones are usually made of nickel.

Electrostatic lenses (Figure 2-15 D) keep the ions focused in a compact "ion beam" as they pass through the vacuum system to the final chamber, where the mass spectrometer and detector are housed. The ion lenses perform a second, essential, function of separating the ions from the photons and residual neutral material.

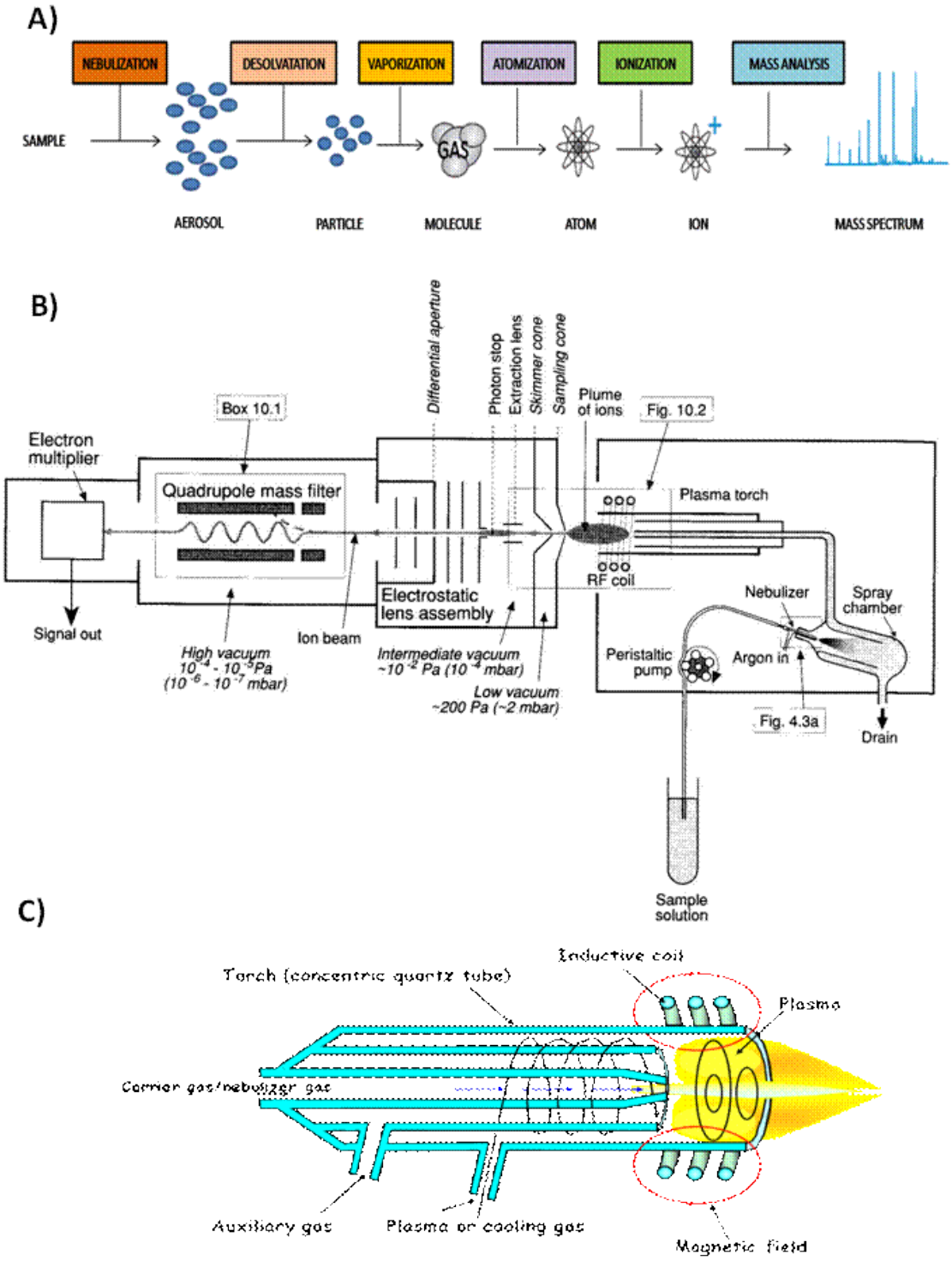


Figure 2-14: A) processes involved into ICP-MS analysis adapted from (Agilent, 2005). B) structure of an ICP-MS and of a plasma torch (C). B and C are kindly provided by Prof. Joerg Feldmann.

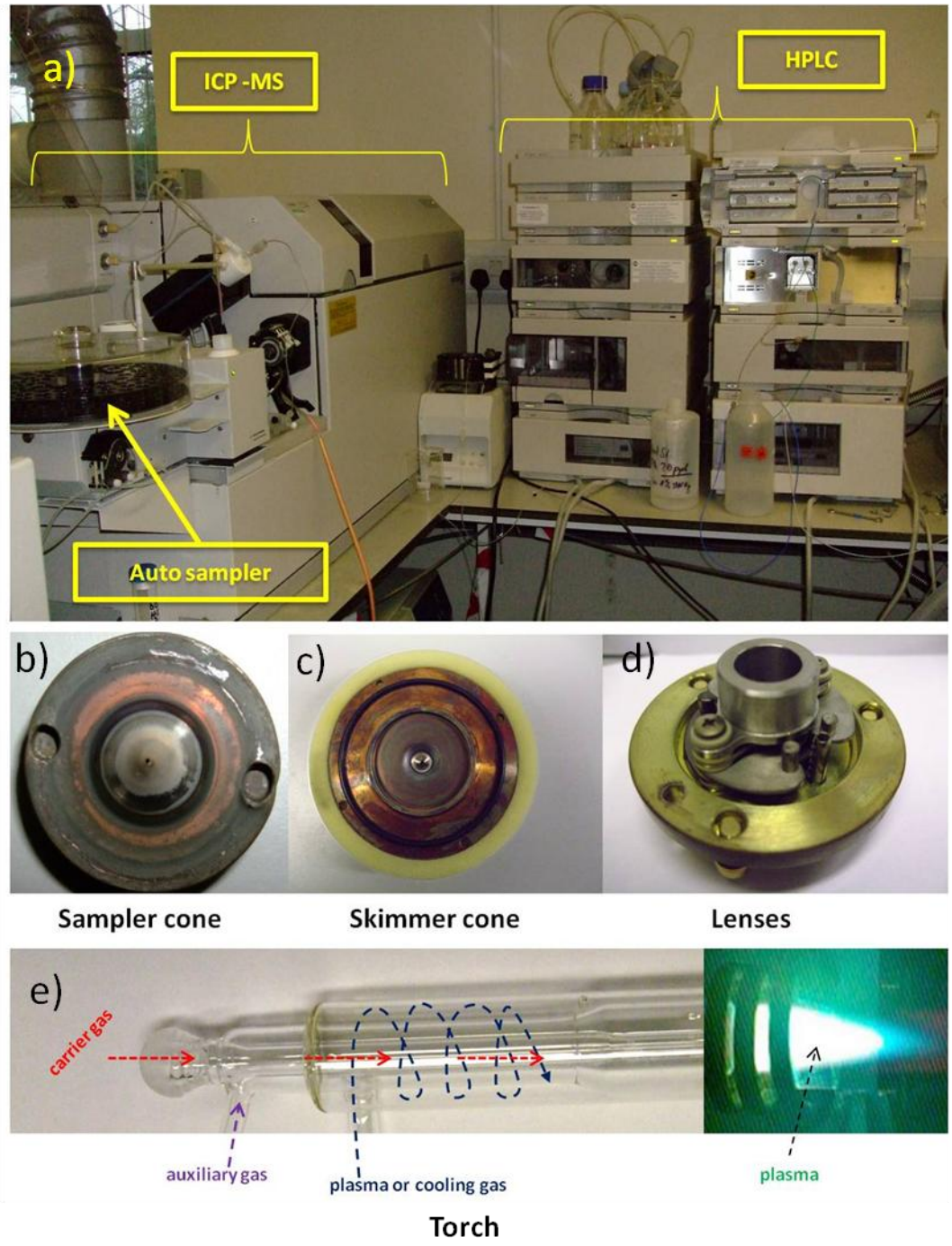


Figure 2-15: A) Agilent 7500 ICP MS. It can be coupled to the HPLC. B) sampler cone, C) skimmer cone, D) lenses and E) torch.

Three different types of mass analyzers have been used with ICP-MS: quadrupole, magnetic sector, and time-of-flight analyzers. The most common mass analyzer used in ICP-MS is the quadrupole that separate ions based on their mass to charge ratio (m/z). Since the plasma produces almost exclusively singly-charged ions, the mass/charge ratio is equal to the mass of the ion, making the spectrum very simple to interpret.

A collision/reaction cell system can be used to remove spectral interference in ICP-MS. The device consists of an ion guide, which is enclosed in a cell that can be pressurized with a gas and is located after the main ion lens (Agilent, 2005). The gas interacts with the ion beam to remove polyatomic interference in two possible ways: the gas itself reacts with the interference and converts it in a different species (reaction mode); the gas collides with the polyatomic interference causing its loss of energy (collision mode). The most commonly used gases for collision/reaction cells are H_2 and He. Rarely, methane is used.

4 .2 Interferences in ICP-MS

Even though ICP-MS offers a range of advantages to identify and quantify the elemental composition of a biological sample, it is affected by interferences that represent a limitation of the technique.

Isobaric interferences arise from the direct overlap from a different element with an isotope at the same nominal mass (Agilent, 2005) (i.e. ^{58}Fe on ^{58}Ni). Molecular or polyatomic interferences, are due to the formation of molecular compounds such as oxides. As an example, when analysing urine that is a complex biological matrix, the signal of argon chloride ($^{40}Ar^{35}Cl$) occurs on ^{75}As, and overlaps it. This can cause an overestimation of arsenic content and misleading results. In order to overcome this problem a collision/reaction cell technology can be used as explained in section 4-1 of this chapter.

Another type of interference is the non-spectral interference that is defined as a matrix inducted signal variation (enhancement or suppression) and it is referred as “matrix effect”.

Most of clinical samples suffer from interferences deriving from relatively high concentration of organic species. Some examples of known interferences in urine are reported in Table 2.9. In order to solve this issue, along with the use of a collision cell, the

interference free-isotope is usually preferred (Hsiung *et al*., 1997). In the case of copper, ^{63}Cu suffers from interference with $^{40}Ar^{23}Na$ while interference with S and Ca species have a relatively low contribution to mass 65 that has been preferred. For zinc, the isotope ^{68}Zn is preferred to ^{67}Zn and ^{66}Zn, because relatively free from S and O species interferences in urine (Hsiung *et al*., 1997).

Table 2.9: Some isotopes analysed in urine using ICP-MS and possible polyatomic interferences on specified mass modified from(Townsend *et al.,* 1998).

Isotope	Abundance (%)	Possible Polyatomic Interferences
^{63}Cu	69.17	$^{40}Ar^{23}Na$, $^{23}Na2^{16}O1H$, $^{23}Na_2{}^{17}O$, $^{31}P^{16}O_2$, $^{35}Cl^{14}N_2$
^{65}Cu	30.83	$^{33}S^{16}O_2$, $^{32}S^{16}O^{17}O$, $^{32}S^{33}S$, $^{32}S^{16}O2^{1}H$, $^{48}Ca^{17}O$, $^{48}Ca^{16}O^{1}H$, $^{37}Cl^{14}N_2$, $^{31}P^{16}O^{18}O$, $^{31}P^{17}O_2$
^{66}Zn	27.90	$^{32}S^{16}O^{18}O$, $^{32}S^{17}O2$, $^{33}S^{16}O^{17}O$, $^{33}S^{16}O2^{1}H$, $^{34}S^{16}O_2$, $^{33}S_2$, $^{32}S^{34}S$, $^{48}Ca^{18}O$, $^{48}Ca^{17}O^{1}H$
^{67}Zn	4.10	$^{33}S^{17}O2$, $^{33}S^{16}O^{18}O$, $^{32}S^{17}O2^{1}H$, $^{32}S^{16}O^{18}O1H$, $^{34}S^{17}O^{16}O$, $^{33}S^{34}S$, $^{36}Ar^{31}P$, $^{35}Cl^{16}O2$
^{68}Zn	18.80	$^{36}S^{16}O2$, $^{34}S^{16}O^{18}O$, $^{34}S^{17}O2$, $^{32}S^{18}O_2$, $^{33}S^{17}O^{18}O$, $^{32}S^{36}S$, $^{34}S2$, $^{36}Ar^{32}S$, $^{35}Cl^{16}O^{17}O$, $^{35}Cl^{16}O_2{}^{1}H$, $^{36}Ar^{16}O_2$, $^{40}Ar^{14}N_2$
^{111}Cd	12.80	$^{95}Mo^{16}O$
^{114}Cd	28.73	^{114}Sn, $^{96}Mo^{16}O$

4 .3 Hyphenated techniques for arsenic speciation

Hyphenated techniques allow the coupling of two on-line methods for the simultaneous qualitative and quantitative evaluation of the arsenicals in a sample. This approach is widely applied to the speciation analysis of metals and metalloids in urine and especially for arsenic and selenium (Lindberg *et al.*, 2007a, Newcombe *et al.*, 2010, Gammelgaard *et al.*, 2005, Hata *et al.*, 2007, Morton and Mason, 2006, Pearson *et al.*, 2007, Ritsema *et al.*, 1998).

During a speciation analysis three basic processes take place: i) extraction, ii) separation, and iii) selective detection of species. Different coupling combinations (separation + element-selective detection) have been attempted in the literature for arsenic speciation

analysis in urine as briefly show in Table 2-10. Among the separation methods, High-Performances Liquid Chromatography (HPLC) is one of the more commonly used. HPLC can be coupled to various spectroscopic methods for analysis of arsenic in urine samples. The combinations include Hydride Generation Atomic Absorption Spectroscopy (HPLC-HG-AAS), which has been applied to arsenic speciation in urine for AsIII, AsV, MMAV, and DMAV (Tsalev et al., 2000, Le and Ma, 1997, Howard and Hunt, 1993). HPLC-Hydride Generation Atomic Fluorescence Absorption Spectrometry (HPLC-HG-AFS) has also been applied to arsenic speciation in urine (Corns *et al*., 1993). It has been reported through a inter-comparison of analytical methods for arsenic speciation that there is no systematic difference in the speciation of four arsenic species (AsIII, AsV, MMA and DMA) measured using AAS and ICP-MS (Crecelius and Yager, 1997). However, HPLC-ICP-MS, can be considered as the most sensitive and robust and commonly used method for arsenic speciation analysis in diverse matrices (Caruso and Montes-Bayon, 2003).

Table 2-10: Hyphenated techniques applied for human biological samples speciation

Separation method	+	Element-selective detection	Reference
HPLC: - Reverse Phase - Ion Exchange		ICP	(Caruso and Montes-Bayon, 2003)
Gas Chromatography		ICP	(Mester and Pawliszyn, 2000)
HPLC		HG-AAS	(Tsalev *et al*., 2000)
HPLC		HG-AFS	(Corns *et al*., 1993)

In order to physically connect the separation column with the detector, a narrow-bore tube from the liquid chromatographer is introduced into the nebulizer of the ICP-MS. Since the discharge eluent from the chromatographer may be between 0.1 and 1 ml/min and the uptake rates for micro- macronebulizers of the ICP-MS are the same (Caruso and Montes-Bayon, 2003), the connection between the two devices is possible. However, there are compatibility limitations related to mobile phase conditions such as the presence of organic solvents, salts and buffers that can produce clogging of the cones (of the ICP-MS) and/or changes in sensitivity.

After separation of different chemical species on the separation system, the identification of arsenicals is reached by chromatographic comparison (retention time) with specific standards. The specificity of the method depends on the availability and stability of

standard compounds. Errors in the identification can arise from coelution of species or from non full characterization of second products of reaction in standards synthesis (Beauchemin, 2002). Therefore, confirmation by other techniques such as electrospray ionization (ESI) should be highly convenient.

Different liquid chromatography techniques have been used for the separation of the As species prior to the detection via ICP-MS. The studies presented in this thesis focus only on the most used and performing techniques applied for arsenic speciation in urine.

4. 4 Reversed-phase and Reverse-phase Ion pairing (RPIP) Chromatography

Reversed phase Chromatography (RP-HPLC) can be successfully coupled to ICP-MS for determination of arsenic species in urine (Dopp et al., 2008).

In RP-HPLC a non-polar stationary phase (normally octadecyl, C18 or octyl C8 chains) is bonded to a solid support that is generally microparticulate silica gel. The mobile phase is polar. The analytes are partitioned between the mobile and the stationary phase. The separation is normally performed using aqueous mobile phases containing different percentages of organic modifiers (e.g. methanol, ethanol, acetonitrile, or THF) to increase the selectivity between species. Solute retention is also influenced by eluent pH, which affects the dissociation level of the analyte and therefore, its partition between the mobile and the stationary phases.

RPIP-HPLC is a special mode of RP-HPLC used for the separation of ionic or ionizable compounds for which an ion-pair is formed between the solute ion and an appropriate ion of the opposite charge (the counter ion present in the mobile phase). The resulting ion-pair is hydrophobic and it is partitioned between the mobile and the stationary non-polar phase so, the separation is based on the "ion-pairing" polarity.

Mobile phases are similar to the ones used in RP-HPLC but with the addition of an ion-pairing reagent (like tetraalkylammonium salts and alkylsulfonates).

The main limitation of RP-HPLC–ICP–MS is that most organic modifiers are not ICP–MS compatible. In fact, only low percentages of MeOH or EtOH can be run in ICP–MS instruments using conventional sample introduction devices (such as the concentric nebulizer) without seriously compromising the sensitivity. The use of micronebulizers has

permitted the introduction of other organic modifiers, such as acetonitrile in conjunction with microbore columns (Acon et al., 2001).

4.5 Ion-exchange Chromatography

Ion exchange chromatography (IEC) is based on the interactions of charged analytes (anions or cations) with the charged (positively or negatively) functional groups of the stationary phase. The mobile phases usually employed consist of an aqueous salt buffer solution (such as phosphate buffer), often mixed with a certain amount of organic modifier, such as methanol or acetonitrile.

Gradients allow the separation of complex mixtures but they present two major drawbacks when used in HPLC–ICP–MS separations: they are time consuming (once the run is finished, the column needs to equilibrate with the new mobile phase) and the changes in the composition of the mobile phases during the run may lead to changes in the background or sensitivity. Additionally, high saline concentrations are undesirable for an adequate ICP–MS performance as described in previous sections. However, most separations carried out using IEC involve the use of gradients to increase ionic strength during the chromatographic run.

IEC is the most used chromatography technique in urine speciation analysis. Different authors report different performances in terms of peaks resolving with different columns/conditions/mobile phase. For instance, Brima *et al.* (Brima *et al.*, 2006a) separated AB, MA (V), As (III), As (V) and DMA(V) using as mobile phase 20mM NH_4HCO_3 (5% methanol), pH 10.3 on an anion exchange column Hamilton PRP-X 100 according to the method developed by (Pedersen and Francesconi, 2000).

Todorov *et al.* (2005) separated five arsenic species in urine using as a mobile phase 4mM ammonium hydrogen carbonate, 4mM ammonium sulphate, and 4mM ammonium phosphate (pH 8.9) as mobile phase on a PRP-X100 (Hamilton, Reno, NV) anion exchange column.

Feldmann (1999) used a strong anion-exchange column with 30 mmol/L phosphate buffer as the mobile phase (pH 6.0) for the separation of As(III), DMA, MMA, and As(V) (see Figure 2-16). A similar approach has been used in this thesis to speciate arsenicals in urine, as detailed in the methods section of Chapter 3.

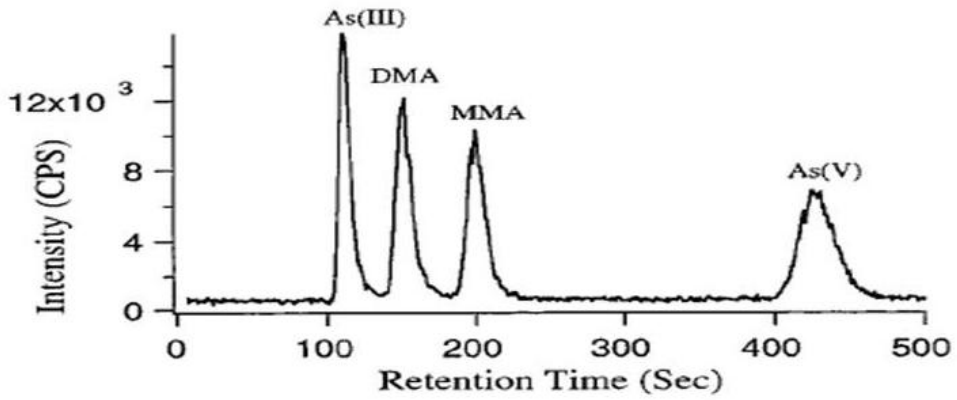


Figure 2-16: Urinary chromatograms resolving main arsenicals on an IEC column from (Feldmann *et al.*, 1999).

In order to separate AB in urine, a cation-exchange chromatography can be used with 20 mmol pyridine (pH adjusted to 2.7 with formic acid) as a mobile phase. Recently, Morton and Leese (Morton and Leese, 2011) developed a new method for arsenic speciation in urine based on micro-liquid chromatography using a low-pressure delivery six-port valve with a 5 cm anion exchange column that allows a fully resolved separation of five arsenic species (AB, AsIII, AsV, MMA(V) and DMA(V)) in urine in just 6 minutes.

To separate and quantify thioarsenicals and trivalent methylated species in urine, comparison with standard retention time is not the best method for peak identification. In this case, to identify arsenicals, it is recommended to couple Electrospray Ionization Mass Spectrometry (ESI-MS) to HPLC in order to provide solid data on identification and quantification. The level of rigour required for identification will depend on the species and type of sample (Hansen et al., 2004).

4.6 Urine preparation and quality controls in ICP-MS

From an analytical point of view, urine is a complex matrix, rich in sodium chloride, other salts and organic carbon and it is considered to be a "dirty sample". Urine analysis with ICP-MS is affected by both isobaric interferences and non-spectral interferences. These interferences can result in the suppression or enhancement of the analyte signal. High levels of chlorine in the sample can drastically limit detection.

The use of a higher dilution factor is a possible solution that minimizes the matrix effect. On the other hand, if the average analyte concentration in the sample is relatively low, a too high dilution factor can result in too many samples falling below the limit of detection of the method. Therefore, a compromise has to be found to be able to maximise the throughput, minimise the eventuality of contamination from dilution and counter interferences. Some authors filter urine before analysis (Lintschinger *et al.*, 1998; Shibata *et al.*, 2005). Different types of diluents can be used for urine but essentially it has to match the composition and %acid concentration of the one used for the standard to minimise signal drift because of a matrix effect.

Hydride generation has also been used to minimize chloride interference. The advantages of this HG-ICPMS system compared with ICP-MS detection with direct nebulization, are the increase in sensitivity, the decrease in matrix interferences and additional selectivity because the system can distinguish hydride-active from non-hydride active arsenic species (Francesconi and Kuehnelt, 2004). Increased instrumental complexity and reagent contamination (mainly from $NaBH_4$) are known as the main disadvantages of the HG system (Francesconi and Kuehnelt, 2004).

Another method to avoid chloride interference was developed by Ritsema *et al*. (1998) which uses a chromatographic system with an anion-exchange column and gradient elution of ammonium carbonate buffer to elute chloride after the elution of all the arsenic species.

One of the possibilities for reducing the impact of the organic carbon in urine is to perform a mineralization of the organic matter to elements by means of a microwave reactor. The heating induced by the microwaves occurs via molecular agitation of the dipoles of the water molecules (Rouessac and Rouessac, 2007).

If analyte suppression is not too great and the sample matrix composition is known, an external calibration can be used**.** An internal standardization to compensate for small matrix differences can be also effective (Rodushkin *et al.,* 1999). Ideally, the internal standard should be similar in mass and ionization potential to the analytes. However, depending on the matrix effect, one similar in mass may be quite effective if space charge effects predominate (Baker *et al.,* 1999).

The method of standard addition (MSA), which is usually more time-consuming in terms of sample preparation, is carried out to obtain accurate results. For MSA, due to the wide linear dynamic range of ICPMS, a single large, multi-element spike can be used to obtain accurate results, making sure that the resulting analyte signals fall near the upper end of the dynamic range (Abbyada *et al.,* 2001). Because the spike does not have to be matched to the analyte concentration, this simplified procedure eliminates the preliminary analysis that is normally required. Furthermore, a correction for linear drift could be achieved by bracketing the unspiked sample immediately before and after the spiked one in the sequence of measurement (Abbyada *et al.,* 2001). However, this drift correction method may be problematic for elements that are prone to memory effects.

An internal standard is used to account for signal drift in the plasma. A known amount of an element that is not present in the sample composition (rare elements as rhodium, indium or palladium) are added to each sample, to the blanks and to the external standards at a fixed concentration (usually 10 ppb). This addition can nowadays be performed with an online tubing system before the sample reaches the plasma (Winkel *et al.*, 2009).

The use of Certified Reference Materials (CRMs) is the standard way to ensure the quality of the method in use. Different products are nowadays commercially available for biological samples (urine, hair, plasma, whole blood) including both certified products for total trace elements and arsenic speciation. For urine, products are sold as freeze dried powder to be reconstituted in a exact amount of ultrapure water before the analysis. Seronorm trace elements in urine were used in this study (Veillon and Patterson, 1996) certified for a range of total trace elements. Additionally, NIES CRM No.18 Human Urine produced by the National Institute for Environmental Studies (NIES), Ibaraki, Japan was used as well.

The quality of the analytical method is assessed by calculating the % recovery expressed as

$$\% \text{ recovery CRM} = 100 \text{ X } [Qa]/ [Ca] \quad \text{(Equation 4)}$$

Where

Qa = concentration of the experimentally detected analyte
Ca = concentration of the certified analyte

Finally, in order to minimise the impact of a possible memory effect on the analysis, it is important to randomise the order or the samples during the ICP run and to have a 10% of replicated prepared samples.

5. "-Omics" approach for the study of biomarkers of arsenic

The traditional evaluation of human exposure to trace elements is based on the analytical quantification of the element (or its metabolites) in a biological specimen that acts as a biomarker of past or recent exposure. However, as discussed in the first paragraph of this Chapter, the exposure to a xenobiotic can result in the alteration of normal biochemistry of a cell, organ or organism. Changes can be detected in biofluids in order to monitor the biochemical and biological effects.

Along with the traditional approach, the application of "-omics" techniques to investigate biomarkers of sensitivity and effects of arsenic (Ghosh and Poisson, 2009) and other trace elements, and more often heavy metals, is an emerging field of research.

The term '-omics' is used to indicate genomics (and transcriptomics), proteomics and metabolomics. Figure 2-17 shows some of the "-omics" techniques that can be applied to detect biomarkers of arsenic effects and sensitivity. -Omics are currently broadly applied in medicine, systems biology and molecular diagnostics to look for new biomarkers of disease. Very little has been done in the arsenic field in particular and for other trace elements.

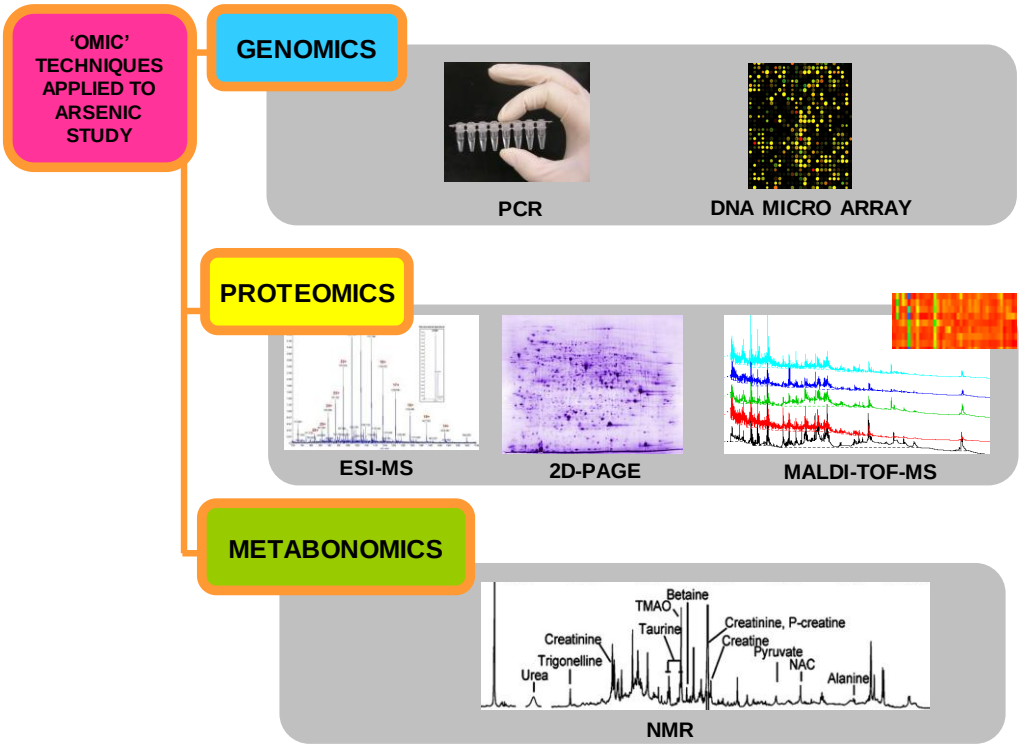


Figure 2-17: Examples of possible 'Omics' techniques applied to arsenic study

5.1 Genomics in assessing arsenic risk

Genomics deals with the analysis of the genome that is the full amount of genes present in a certain organism. Genomics studies include the investigation of the mutations resulting in pathological outcomes. The human genome consists of 20-25,000 genes and it was fully sequenced in 2003 with the completion of the Human Genome Project (http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml).

After completing the sequencing of the human genome, scientists realised that most of the information required to understand how DNA interacts to external *stimuli* was still missing. In fact, alternative splicing and post-transcriptional modifications of proteins play a role in the final expression levels of a protein and the paradigm "one gene - one protein" is no longer considered valid. Therefore, genomics *per se* is not sufficient to understand how organisms interact with the environment and develop pathological and/or physiological responses to it.

Several studies on arsenic have already used *genomics* as an investigative tool to monitor changes in gene expression (Ghosh *et al.*, 2008), individual susceptibility and

biomethylation pathways and toxicity related to Single Nucleotide Polymorphisms (SNPs) in crucial genes. Some examples of genomics and transcriptomics[8] techniques applied to investigate arsenic genotoxicity *in vivo* and *in vitro* are shown in Table 2-11. For instance Hsu *et al.* (2008) demonstrated the relationship between arsenic exposure and chromosomal aberrations in biopsies of patients affected by urinary transitional cell carcinoma in a study group from Taiwan, by means of comparative genomic hybridization (CGH). Furthermore, Table 2-12 reports polymorphisms in certain genes associated with arsenic exposure.

Table 2-11 : Genomics and transcriptomics applied to the study of arsenic exposure and effects

Technique	Application	Organism	Reference
DNA microarray	Study of DNA methylation and gene expression changes in lung following subchronic arsenate exposure	*In vivo*, mouse	(Boellmann *et al.,* 2010)
Comparative genomic hybridization (CGH)	Study of association between arsenic exposure and chromosomal aberrations in patients affected by urinary transitional cell carcinoma	humans	(Hsu *et al.,* 2008)
Single nucleotide polymorphism (SNPs)	Association studies to explore relationships between SNPs and arsenic methylation capacity and health impact	humans	See Table 2-12
Micronuclei (MN)	Arsenic exposed individuals showed a statistically significant increase in the frequency of MN in oral mucosa, urothelial cells and lymphocytes	humans	(Basu *et al*., 2002)

Finally, a recent study (Smeester *et al.*, 2011) enabled a comprehensive examination of DNA methylation levels within CpG islands[9] for over 14,000 genes in a iAs study group (*n*=16) and demonstrated that a large number of genes are epigenetically modified in the lymphocyte DNA of individuals exposed to iAs with related arsenicosis. A total of 183 genes were found with differential methylation patterns and 182 were hypomethylated in volunteers showing signs of arsenicosis. Using a systems level approach, 183 genes were

[8] the study of the mRNA of a cell, an organ or an individual in a certain moment of its life

[9] The CG island is a short stretch of DNA with high CG frequency. It is also called the CpG island. CpG islands are often located around the promoters of **essential genes** or other genes frequently expressed in a cell. At these locations, the CG sequence is not methylated. By contrast, the CG sequences in inactive genes are usually methylated to suppress their expression.

Table 2-12: Polymorphisms associated with arsenic exposure modified from (Ghosh et al., 2008),

Gene	Reported Polymorphism	Study population and location	Association with	Odd Ratio (95%CI)	Reference
GSTT1	Gene deletion	Pabna, Bangladesh	arsenic induced skin lesions	1.56 (1.1–2.19)	(McCarty *et al.,* 2006)
GSTM1	Gene deletion	West Bengal, India	arsenic induced skin lesions	1.73 (1.24 – 2.22)	(Ghosh *et al.,* 2006)
GSTP1	(A > G) Ile105Val	Pabna, Bangladesh	increased arsenic toxicity	1.86 (1.15–3.00)	(McCarty *et al.,* 2006)
GSTP1	(A > G) Ile105Val	South West Guizhou, China	increased arsenic toxicity	4.72 (2.34–9.54)	(Lin *et al.,* 2006)
PNP	(G > A) Gly51Ser	West Bengal, India	arsenic induced skin lesions	1.66 (1.04–2.64)	(Chaudhuri *et al.*, 2008)
p53	(A > G) Arg72Pro	West Bengal, India	increased keratosis	2.086 (1.318–3.299)	(Chaudhuri *et al.*, 2006)
p53	(16bp1/16bp2) Intron 3	West Bengal, India	increased keratosis	2.086 (1.257–3.457)	(Chaudhuri *et al.*, 2006)
ERCC2	(A > C) Lys751Gln	West Bengal, India	increased hyperkeratosis	4.77 (2.75–8.23)	(Banerjee *et al.*, 2007)
ERCC2	(G > A) Asp312Asn	New Hampshire	decreased risk of Squamous Cell Carcinoma	0.8 (0.6–1.0)	(Applebaum et al., 2007)
ERCC2	(G > A) Asp312Asn	New Hampshire	decreased risk of Basal Cell Carcinoma	0.8 (0.7–1.0)	(Applebaum *et al*., 2007)
APE1	(T > G) Asp148Glu	Bangladesh	arsenic induced skin lesions	1.93 (1.15–3.19)	(Breton *et al.,* 2007)
XRCC3	(C > T) Thr241Met	Europe (Hungary, Romania, Slovakia)	decreased risk of Basal Cell Carcinoma	0.73 (0.61–0.88)	(Thirumaran *et al.,* 2006)
NBS1	(G > C) Glu185Gln	Europe (Hungary, Romania, Slovakia)	increased risk of Basal Cell Carcinoma	2.19 (1.23–3.91)	(Thirumaran *et al.,* 2006)

analyzed for known molecular interactions, and a large interactome of hypermethylated genes was identified. These are enriched for their involvement in cancer-associated pathways mediated by genes such as p53. Interestingly, many of the proteins encoded by genes with differentially methylated CpG islands were known players in arsenic associated disease, such as heart disease, diabetes, and cancer. Smeester *et al.* (2011) also identified

an arsenic-methylated tumor suppressorome: a pivotal clue in unravelling a possible epigenetic mode of arsenic-induced disease. The tumor suppressorome is a complex of 17 known or putative tumor suppressors silenced in human cancers. Among the members of the tumor suppressorome, of particular interest are those with known associations to arsenic induced diseases such as cancer of the bladder, kidney, lung, liver, and prostate, as well as cardiovascular disease and diabetes mellitus. This study demonstrates the significant effects of iAs on the epigenome.

5.2 Proteomics in assessing arsenic risk

Proteomics is the study of the proteome. The proteome is the set of proteins and peptides coded by the genome of a cell, organ or organism. Human proteome consists of about 2,000,000 proteins and peptides if we consider isoforms and post translational modifications such as glycosylation and phosphorylation. While the genome is identical in all the cells of an organism, the proteome is peculiar to a cell type and of its specific cell cycle stage. The proteome is therefore a dynamic entity that gives us a snapshot into the life of a cell in a certain moment.

The biochemical composition of biofluids such as urine and blood reflects the changes of an organism in relation to a toxic state or a disease (Barratt and Topham, 2007). Proteomics profiling of biofluids has already been successfully applied for diagnostic and prognostic biomarkers of diseases such as coronary diseases (Delles *et al.*, 2011) or multiple sclerosis (Kroksveen *et al.*, 2011; Lourenço *et al.*, 2011). The detection and comparison of the *urinary proteomic profile* of arsenic from exposed/not exposed individuals can result in the identification of qualitative/quantitative alterations of protein expression as a consequence of arsenic exposure. Urinary proteomics is particularly appealing because urine, compared to other fluids (i.e. blood, serum, cerebrospinal fluid, etc) is a , non-invasive and inexpensive sample. In this context, *urinary proteomics* is an extremely powerful tool for the diagnosis of disease and toxicological response to hazards.

The main techniques applied in proteomics profiling include (see Table 2-13) Matrix-Assisted laser desorption ionization time of flight mass spectroscopy (MALDI-TOF-MS), Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), Electrospray Ionisation (ESI-MS), multidimensional protein identification technology (MudPIT) and Two-dimensional polyacrylamide gel electrophoresis (2D-

PAGE), (Zerefos and Vlahou, 2008; Zerefos *et al.*, 2006; Barratt and Topham, 2007). Proteins and peptides present in urine, or other biological fluids, can be used as fingerprints. Differences found between profiles of exposed and non-exposed subjects can then be utilised to identify candidate proteins as new biomarkers.

Table 2-13: Proteomics techniques for biomarker discovery

Methods for protein separation
• Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) • High Performance liquid chromatography (HPLC) • Capillary electrophoresis (CE) • Multidimensional systems (i.e. MudPIT)
Methods for protein identification
Mass Spectrometry : • Matrix -Assisted laser desorption ionization time of flight mass spectroscopy (MALDI-TOF-MS) • Electrospray Ionization Mass Spectrometry (ESI-MS) Tandem Mass Spectrometry: • Triple quadrupole (Q/Q/Q) • Time of flight-Time of flight (TOF-TOF) • Ion Trap
Protein microarrays
• Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) • ELISA based semi automatised systems • Protein chips • Lab-on chips

A few groups have started to investigate the effect of arsenic exposure on human proteome and recognise that the screening of changes in urinary proteome can be used as an efficient method to establish a panel of potential arsenic-associated pathologies, as well to elucidate the mechanisms involved in chronic arsenic exposure (Navas-Acien and Guallar, 2008). Tan *et al.* (2008), performed proteomic analysis on urine samples from a population in Taiwan exposed to high levels of As(V) in drinking water (350-1140 ppb) by means of HPLC ESI-MS/MS. Urine samples of non-cancer urological patients and patients with either bladder transitional cell carcinoma (TCC) or arsenic-associated bladder TCC, compared to a control population, were analysed. Three urinary proteins were found to have significantly altered levels in patients following chronic arsenic exposure: a disintegrin and metalloprotease protein (ADAM 28) and a calpain 9 and ring finger protein 20. Hegedus *et al.* (2008) conducted urine proteomic analyses on men and women from

Nevada (USA) and Northern Chile, identifying in both study populations a statistically significant decrease in a 4.37-kDa protein in men exposed to high arsenic levels (>500 ug/l) in drinking water compared to men exposed to low levels (<15 ug/l). No statistically significant difference was found in women.

In general, the bottle necks of proteomic approaches are: (i) the generation of large quantities of data that require dedicated computational, mathematical and statistical tools for processing and interpretation; (ii) the presence of confounding factors like inter-individual variability related not to exposure differences but to diet, ethnicity etc; (iii) the need for reliable and rigorous standardized sampling and storage protocols to produce good quality data to share; and (iv) the lack of a database of 'normal' human proteomics profiles for biofluids.

5.3 Metabolomics for assessing arsenic risk

Metabolomics is the comprehensive and simultaneous systematic determination of metabolite levels in a biofluid and their changes over time as a consequence of a stimuli. The name metabolomics was coined in the late 1990s by Oliver *et al.* (1998).

Metabolomics rely on the use of complex technological platforms such as tandem mass spectrometry coupled to an HPLC or another separation method (Wuhrer *et al.,* 2009) and NMR Spectroscopy (Saude *et al.,* 2007). Other techniques applied are reported in Table 2-14.

Table 2-14: Analytical techniques for Metabolomics

Non-optical Spectroscopy:
Nuclear Magnetic Resonance (NMR)
Mass Spectrometry (MS)
Gas Chromatography Mass Spectrometry (GC-MS)
Liquid Chromatography Mass Spectrometry (LC-MS)
Optical Spectrometry:
Fourier Transform Infrared Spectroscopy (FT-IR)
Near Infrared Spectroscopy (NIR)
Raman spectroscopy
Others:
Capillary Electrophoresis (CE)

Metabolomics has mainly been applied in toxicology to assess effects of xenobiotics *in vivo* experiments on rats or other experimental models. Some reported alterations that were identified, thanks to the metabolomic approach, are shown in Table 2-15. The metabolomics approach can help researchers in the identification of measurable biochemical, physiological, or other alterations within an organism that, depending on magnitude, can be recognized as an established or potential health impairment or disease.

Table 2-15: Metabolic markers associated with some toxins, simplified from (Shockcor and Holmes, 2002).

Toxin	**Target organ/toxicity type**	**Associated biomarker**
Cadmium Chloride	Testicular	↑ creatine, ↑ glucose, ↓ citrate
Lead Acetate	Liver/Lung/Kidney	↑acetate, ↑creatine, ↑ glucose, ↑lactate, ↑taurine, ↓citrate, ↓hippurate, ↓succinate, ↑N-acetyls, ↓2-OG.
Mercuric Chloride	Kidney (S3 proximal tubular)	↑acetate, ↑amino acids, ↑ glucose, ↓hippurate, ↓succinate, ↓citrate, ↓creatinine, ↓2-OG, ↑ organic acids.

Metabolomics *sensu strictu,* has not yet been applied in the assessment of health risk from chronic arsenic exposure in humans. However this field can largely benefit from the use of metabolomics. By following changes in the metabolomic profile associated with arsenic exposure in humans it should be possible to monitor biochemical effects on a cell or on a whole organisms.

A few studies have applied metabolomics to demonstrate effects of arsenic exposure on animals including bank voles (Griffin *et al*., 2001). One study on rats (Wei *et al*., 2009) documents urinary, serum and liver metabolomic changes in response to realgar administration (arsenic sulphide based traditional Chinese medication) and found decreased urinary levels of trimethylamine-N-oxide, phenylacetylglycine and hippurate and signs of impairment of amino acid metabolism.

In human based studies however, a certain number of confounding factors can mask the effect of arsenic on metabolomics including diet and ethnicity. These issues are discussed in Chapter 5.

5.4 Magnetic Resonance Spectroscopy

Spectroscopic techniques are so called because they involve the use of electromagnetic radiation to probe atoms. In particular, Nuclear Magnetic Resonance spectroscopy (NMR) uses radiofrequency waves to reveal information about the magnetic nuclei (Bothwell and Griffin, 2010).

NMR spectroscopy is a powerful technique that allows to study molecular and cellular environment of magnetic nuclei such us ^{1}H, ^{13}C and ^{31}P.

An important discriminating feature in NMR is the fact that it detects the nuclei of specific elements looking at their distribution among the molecules of a sample; signals are sensitive to local environment surrounding the nuclei. Moreover, NMR spectroscopy is characterised by high penetration and low damage of the biological sample under analysis. Both *in vitro* and *in vivo* applications of Magnetic Resonance Spectroscopy with the possibility of non-invasive and non destructive analysis of a broad variety of samples have been reported. These include analysis of organ preparations (Brindle and Radda, 1985), tissue samples like tumors, extracts from tissues in solid, liquid or gaseous form (Bothwell and Griffin, 2010). Moreover, Magnetic Resonance Imaging (MRI) is used as a diagnostic tool in a range of pathologies such as multiple sclerosis (McDonald *et al.*, 2001).

The analysis is based on nuclear absorption and emission of radio frequency from the atoms constituting the sample. Sample solutions must be placed in a narrow glass or quartz tubes (see Figure 2-18). The tube is placed into a high field electromagnet whose large coils generate a very strong magnetic field called B_0. Magnetic fields typically range between 2 and 21 Tesla, several hundreds of times stronger than that of the Earth (30-60 mT). A second coil (the probe coil) is able to generate a second electromagnetic field called B_1 field. During data acquisition the sample is irradiated with RF waves generate from the probe coil.

Such RF waves are able to cause the flip of the nuclei of the sample from a ground spin state (-1/2) to the other (+1/2) at higher energy. The more the applied power t, the more

nuclei flip from -1/2 to +1/2. When the B_1 field is switched off, the nuclei relax back to the original state. To do this they emit energy as form of RF wave. The emitted RF waves are detected as Free Induction Decays (FID) and mathematically transformed into frequencies. Then they are presented as one dimensional (1D) (see Figure 2-17) or two dimensional (2D) spectra. The emitted radiation has typically four parameters: 1) intensity, 2) frequency 3), half-life and 4) phase.

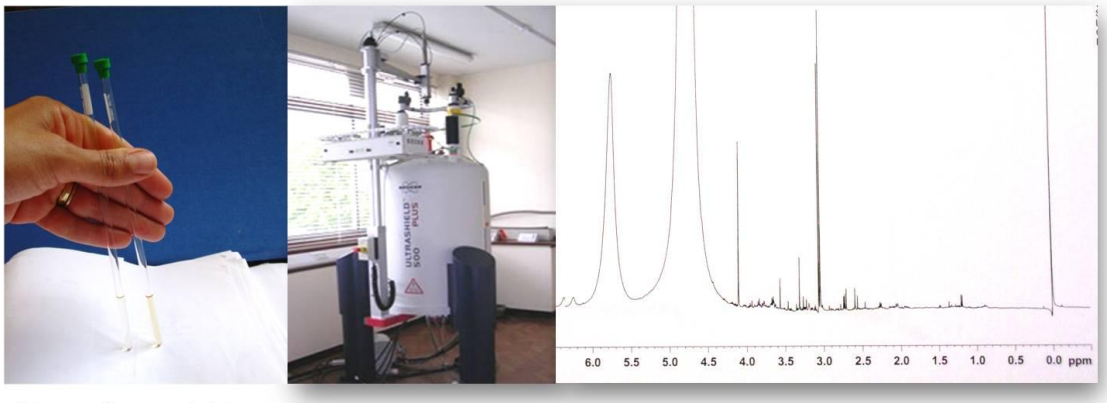


Figure 2-18: Urine prepared for ^{1}H-NMR analysis, Spectrometer used in this study at the Leicester University and a spectra of urine.

In 1D spectra, on the x axis frequency is expressed as chemical shift values in ppm and intensity is on the y axis, as shown in Figure 2-18 and 2-19.

In this thesis, 1D-^{1}H-NMR spectroscopy on urine was used to perform metabolomic profiling. The specific methodology used is described in Chapter 5. Briefly, urine is mixed to a certain amount of an external shift reference such as 3-trimethylsilyl-deuterosodium propionate (TSP) or tetramethylsilane (TMS) in deuterium. On one hand, D_20 allows a reduction in the amount of protonated solvent (such as water), on the other hand, since modern spectrometers work on a 'deuterium locked mode', the deuterium signal is used to calibrate other signals during the experiment. TSP or TMS are used as a reference to set the zero to allow recognition of functional groups on the x axis from the chemical shift values (as shown in Figure 2-19).

Chemical shift values of different metabolites occurring in biological tissues are available in the scientific literature, and the researcher is able to recognise peaks from the position on the x axis. By measuring the area of the peaks and comparing the known concentration

of external standard added in the sample, the quantification can be preformed. This provides the researcher with a characteristic qualitative/quantitative metabolic phenotype from every sample under analysis.

An example of metabolites and relative chemical shift values that can be found in urine is shown in Table 2-16.

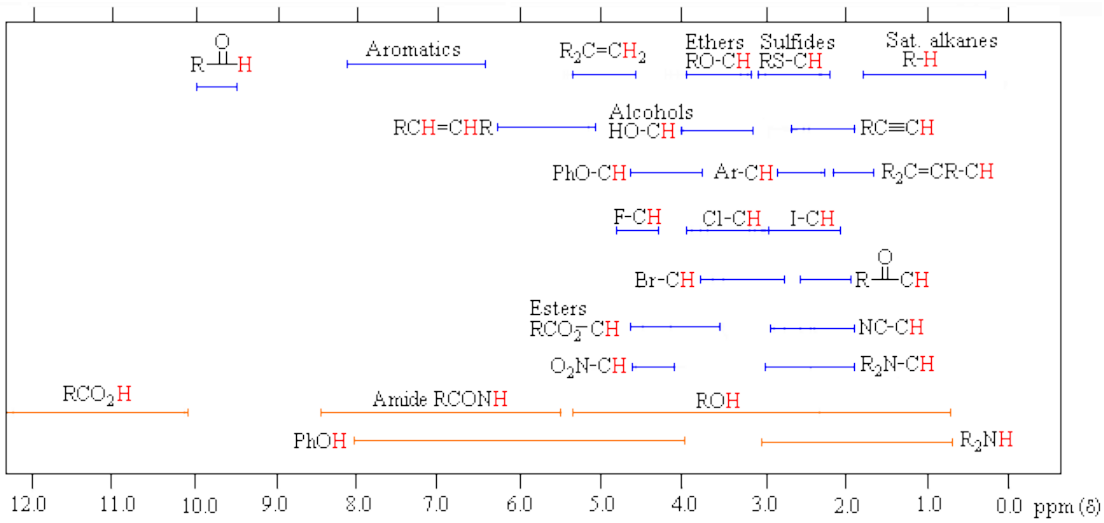


Figure 2-19: Proton chemical shifts for samples in chloroform-d solution. The δ scale is relative to tetramethylsilane (TMS) at δ = 0.
Source
http://www2.chemistry.msu.edu/faculty/reusch/VirtTxtJml/Spectrpy/nmr/nmr1.htm.

Nowadays, commercial software are available to perform spectral pre-processing and peak recognition. For example, Chenomx 7.0 has been used in this thesis. Typical pre-processing steps performed in the Processor module of the software are shown in Figure 2-20. Peaks belonging to urinary compounds are recognised by the chemical shift values comparing to a library for 500 mHz NMR instrument, peaks are fitted (Figure 2-21) and the area automatically calculated by the software. The output will be given to the user as mM or mg/dL for each profiled compound.

The final result of urine sample preparation and analysis is a matrix of concentrations of the n compounds (x variables) for the p samples.

Table 2-16: ^{1}H NMR chemical shift and multiplicity of the functions used to identify metabolites more frequently present in reference group and diabetic urines. DMA is dimethylamine; TMAO is trimethylamine-N-oxide. Table from (Messana *et al.,* 1998).

Metabolite	Chemical shift (ppm)	Function	Multiplicity
3-OH-butyrate	1.21	$[CH_3]$	Doublet
Lactate	1.33	$[CH_3]$	Doublet
Alanine	1.48	$[CH_3]$	Doublet
Acetate	1.93	$[CH_3]$	Singlet
Acetone	2.24	$[CH_3]$	Singlet
Acetacetate	2.29	$[CH_3]$	Singlet
Pyruvate	2.38	$[CH_3]$	Singlet
Succinate	2.45	$[CH_3]$	Singlet
Citrate	2.56 and 2.72	$[CH_2]$	Doublet and doublet
DMA	2.73	$[N(CH_3)_2]$	Singlet
Creatine	3.04	$[NCH_3]$	Singlet
Creatinine	3.06	$[NCH_3]$	Singlet
Carnitine	3.23	$[N^{+}(CH_3)_3]$	Singlet
Betaine	3.27	$[N^{+}(CH_3)_3]$	Singlet
TMAO	3.3	$N(CH_3)_3]$	Singlet
Glycine	3.57	$[CH_2]$	Singlet
Hippurate	7.83	[CH](aromatic)	Doublet-triplet

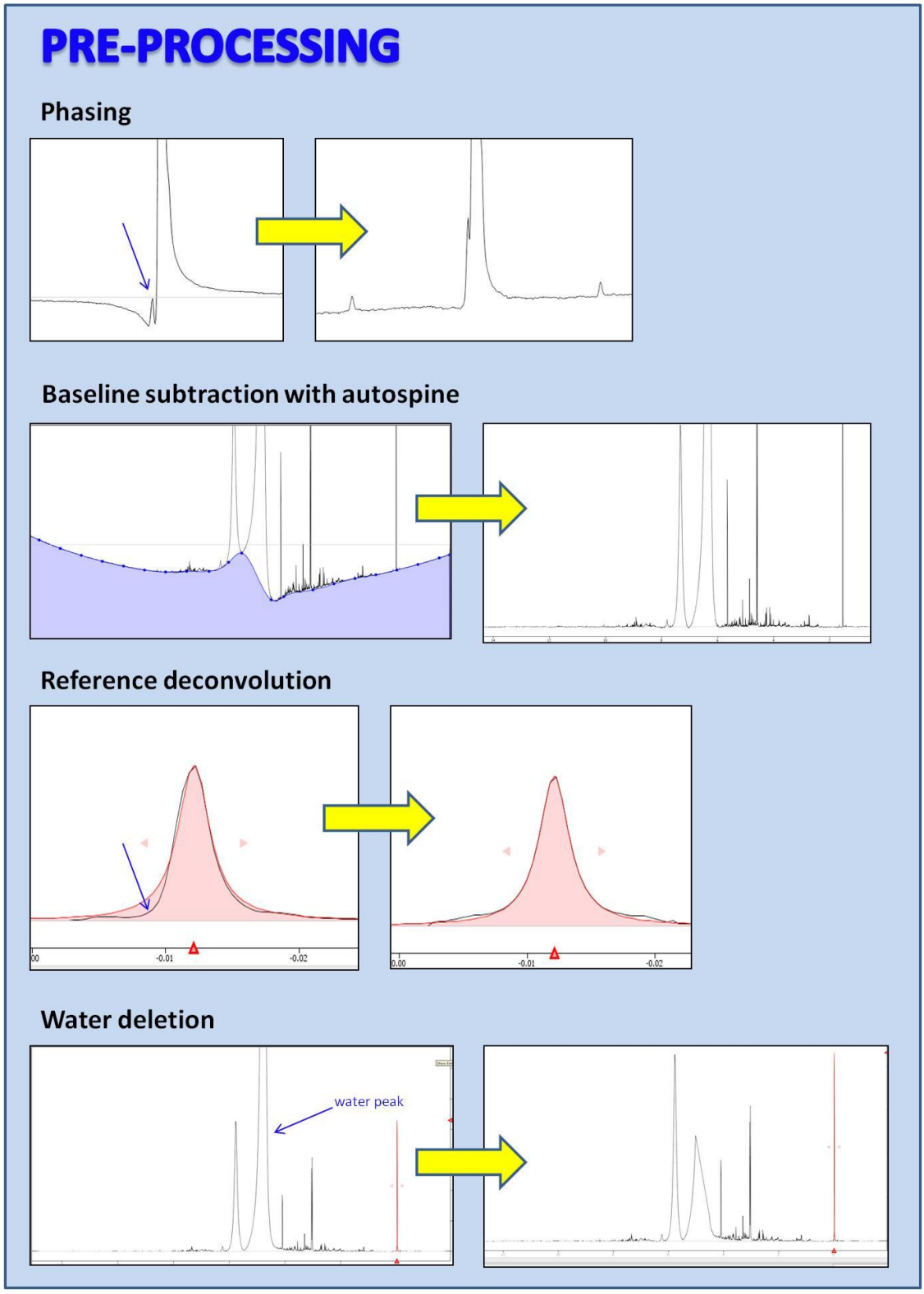


Figure 2-20: Pre-processing steps performed in Chenomx 7.0.

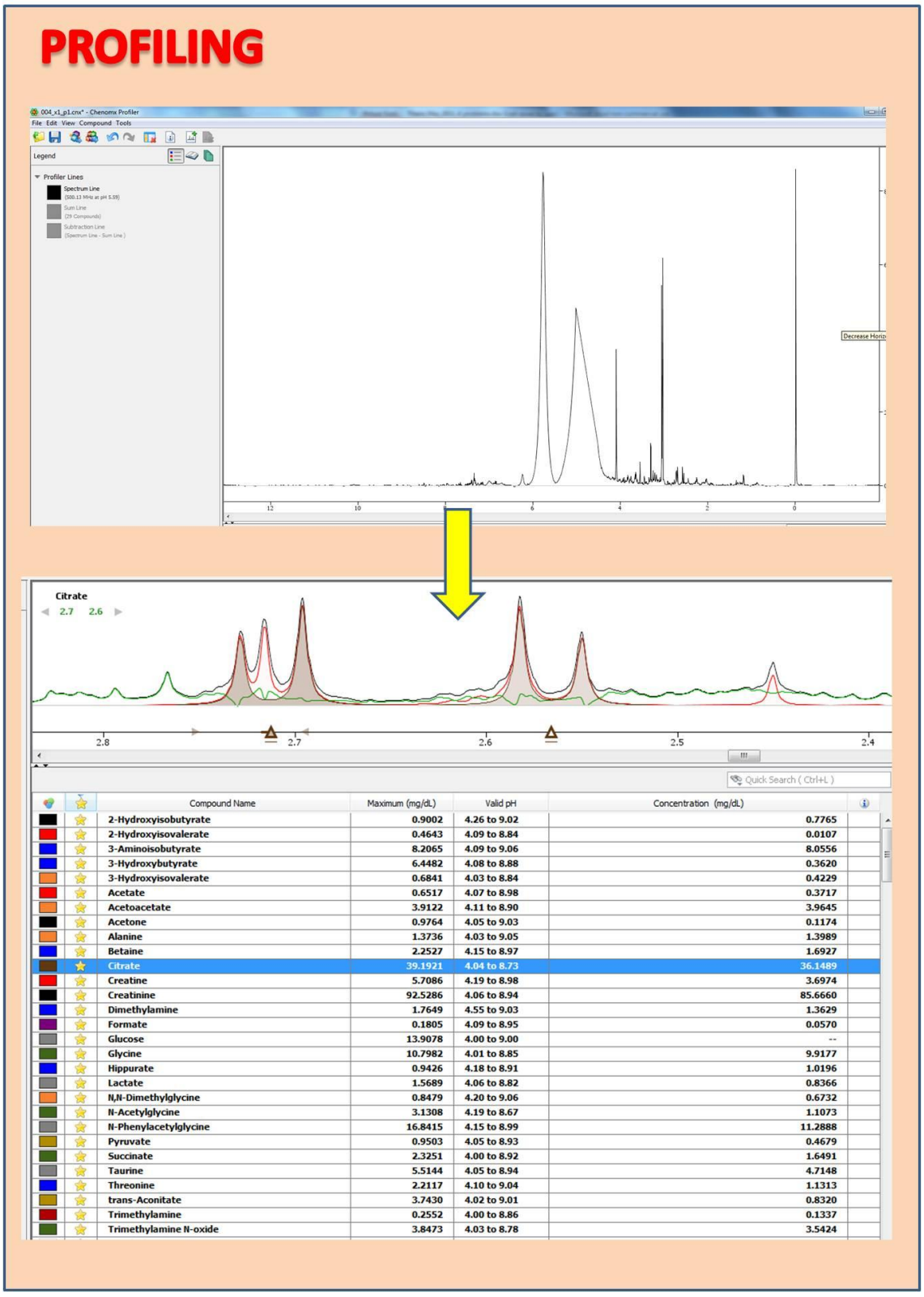


Figure 2-21: Profiling is implemented in Chenomx 7.0 using the 500 mHz library. This allows quantifications of compounds as mM or mg/dL.

6. Data mining and multivariate statistics

The development of a system biology approach that relies on the use of 'high-throughput' techniques generating a large quantity of data, requires a change in research methodology from a classical 'hypothesis driven' one to a 'data generating' one (see Figure 2-22).

'-Omics' techniques (such as 1D-^{1}H-NMR Spectroscopy applied in the present thesis) produce large datasets with up to thousands of variables per single sample. This huge quantity of data contains precious information on the possible biomarkers of a biological event or disease. Multivariate methods are able to handle a variety of variables, reduce the complexity and simultaneously observe changes in relation to the studied phenomenon. However, without the use of a chemometrics approach, such information remains latent and cannot be retrieved from the dataset to gain new knowledge. The term data mining is described by Berry and Linoff (Berry and Linoff, 1997) as "*the exploration and analysis, by automatic or semiautomatic mean, of large quantities of data in order to discover meaningful pattern and rules"*. Such an approach is useful in the aim of screening differences. Figure 2-23 shows a possible methodological pipeline for building new knowledge from large datasets and combining wet lab experiments with *in silico* technologies aimed to discover patterns in the data.

This section does not present the mathematical background to the techniques but presents a 'roadmap' to understand the application of this techniques to practical problems addressed in the thesis.

There are a variety of commercially available resources for data mining (i.e. R, Mat lab). In this piece of work Simca P+ (Umetrics, Umea) was chosen because powerful, versatile and user friendly.

6.1 Unsupervised multivariate approach

Once the dataset has been properly pre-processed (i.e. NMR spectra baseline subtracted, peaks peaked and metabolites identified/quantified), a matrix of data is produced for the p analysed variables (profiled metabolites in urine) as rows and the n observations (samples) as in Figure 2-24.

The first step in data mining is generally devoted to the exploration of the dataset that is performed by means of unsupervised methods. Unsupervised multivariate methods do

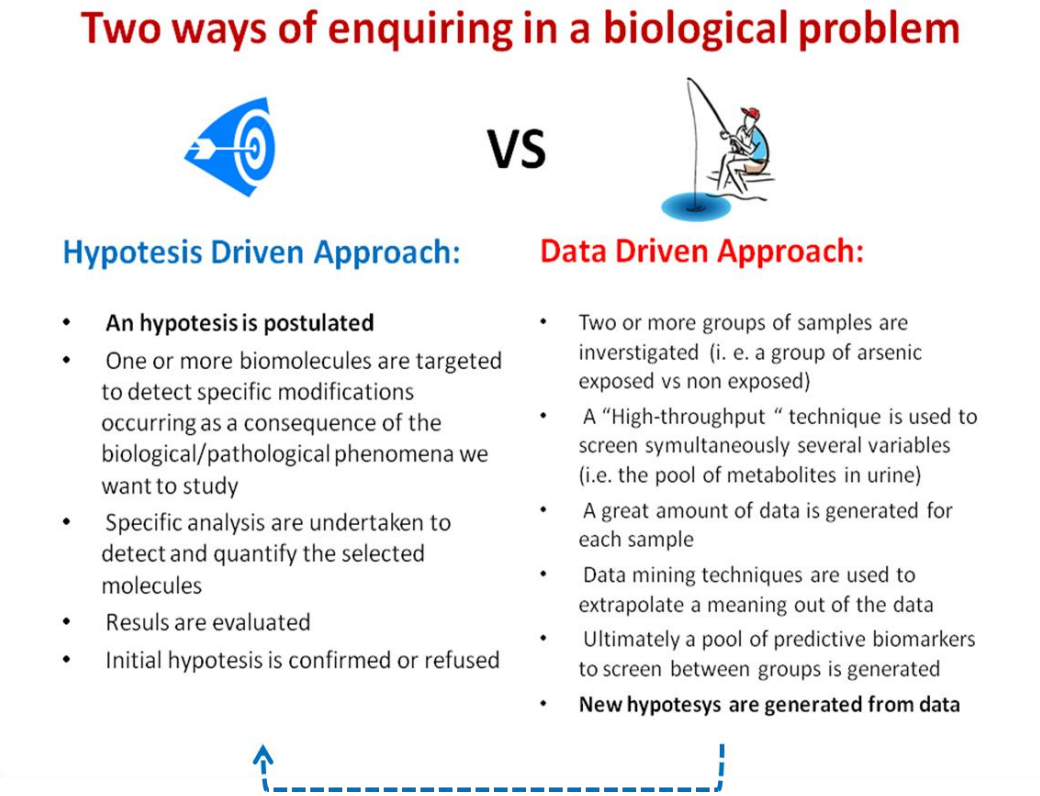


Figure 2-22: Comparison of Hypothesis driven approach versus data driven approach to enquire in a biological problem.

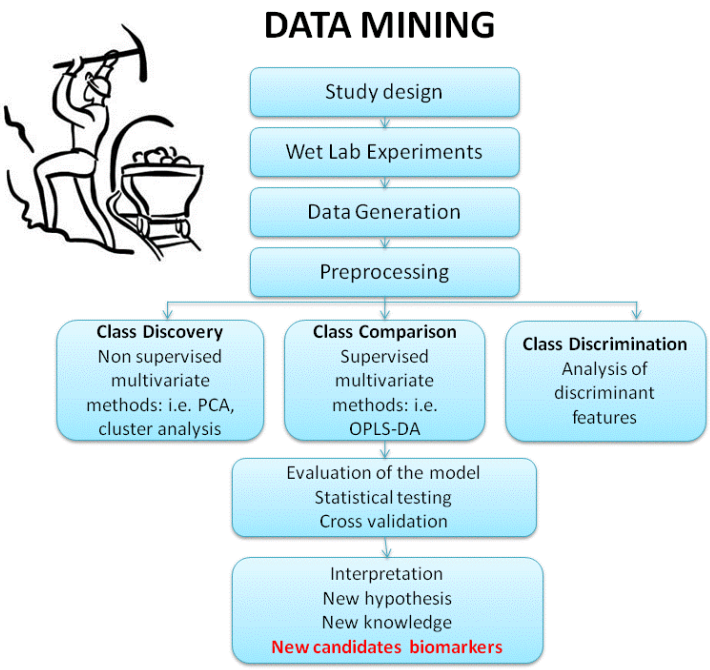
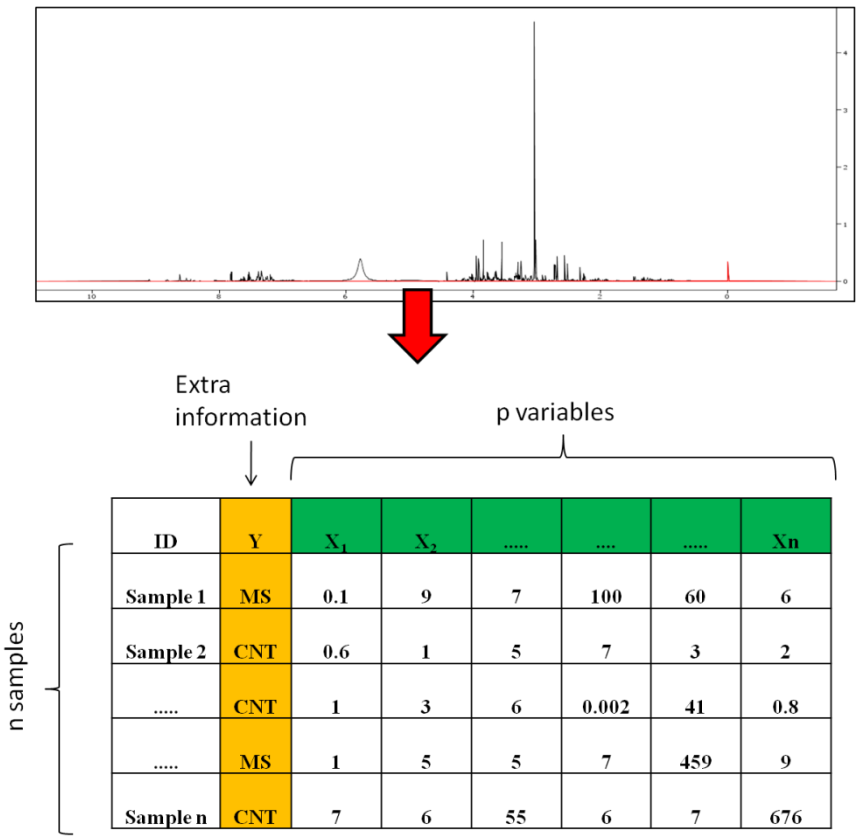


Figure 2-23: Data mining approach for extrapolating meaning from complex datasets, modified from (Dubitzky et al., 2007).



ID	Y	X_1	X_2	.....	....	.....	Xn
Sample 1	MS	0.1	9	7	100	60	6
Sample 2	CNT	0.6	1	5	7	3	2
.....	CNT	1	3	6	0.002	41	0.8
.....	MS	1	5	5	7	459	9
Sample n	CNT	7	6	55	6	7	676

Figure 2-24: From a urinary ^{1}H-NMR spectra to a data matrix. In the pre-processing step, spectra (after normalization procedures) are converted into to a matrix with n samples and p variables; extra information on class (MS: multiple sclerosis patients and CNT: control) can be used as Y variable.

Not require *a priori* knowledge of the sample class (i.e. arsenic exposed *vs* non exposed) and is devoted to explore the 'natural' patterns present in a dataset. Principal Component Analysis (PCA) (Hotelling, 1933, Pearson, 1901) is one of the most widely used method for data mining. The central idea of PCA is to reduce the dimensionality of a data set consisting of a large number of interrelated variables, retaining as much as possible of the variation present in the dataset. This is reached with a mathematical transformation into a new set of uncorrelated variables, or the principal components (PCs). The PCs are ordered in such a way that the first few retain most of the variation present in all of the original variables (Jolliffe, 2002). The greatest part of the dataset variability is explained by the first 3 PCs, and will show systematic trends within the data, i.e., clustering, time trends.

The easiest way to understand the output of a PCA model is to examine the biplot (Figure 2-25) where the n samples are represented by points in a Cartesian plane where the axes are the PC1 and PC2 (or PC3). In the biplot, original variables, p, are represented by vectors that provide an indication on the contribution to the principal component and data separation along the PCs.

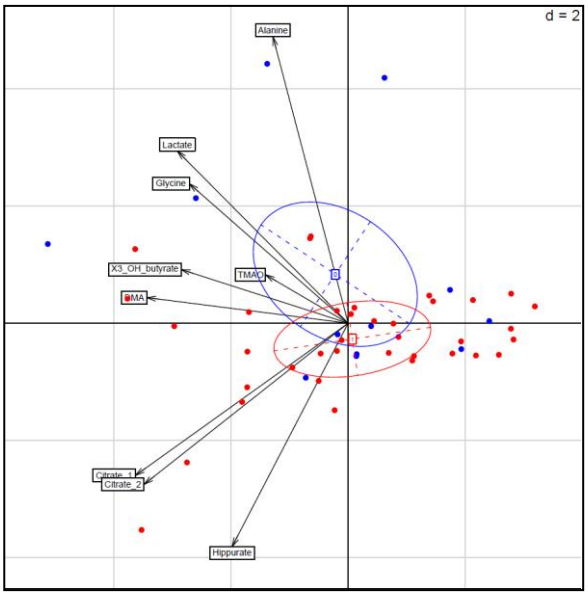


Figure 2-25 Biplot derived from a PCA analysis

The unsupervised approach is useful for preliminary exploration, but it is not specifically able to address the variance related to class separation (for instance affected *vs* not affected by a condition as in Figure 2-26). Unsupervised methods account for experimental drifts, systematic errors and unknown factors playing roles as confounding factors and masking the variation specifically related to the condition under study.

6.2 Supervised multivariate approach

In supervised methods, the *a priori* information, (e.g., object membership) constitutes an extra data table, *Y*, where the columns indicate sample information, which can be a discrete or continuous value.

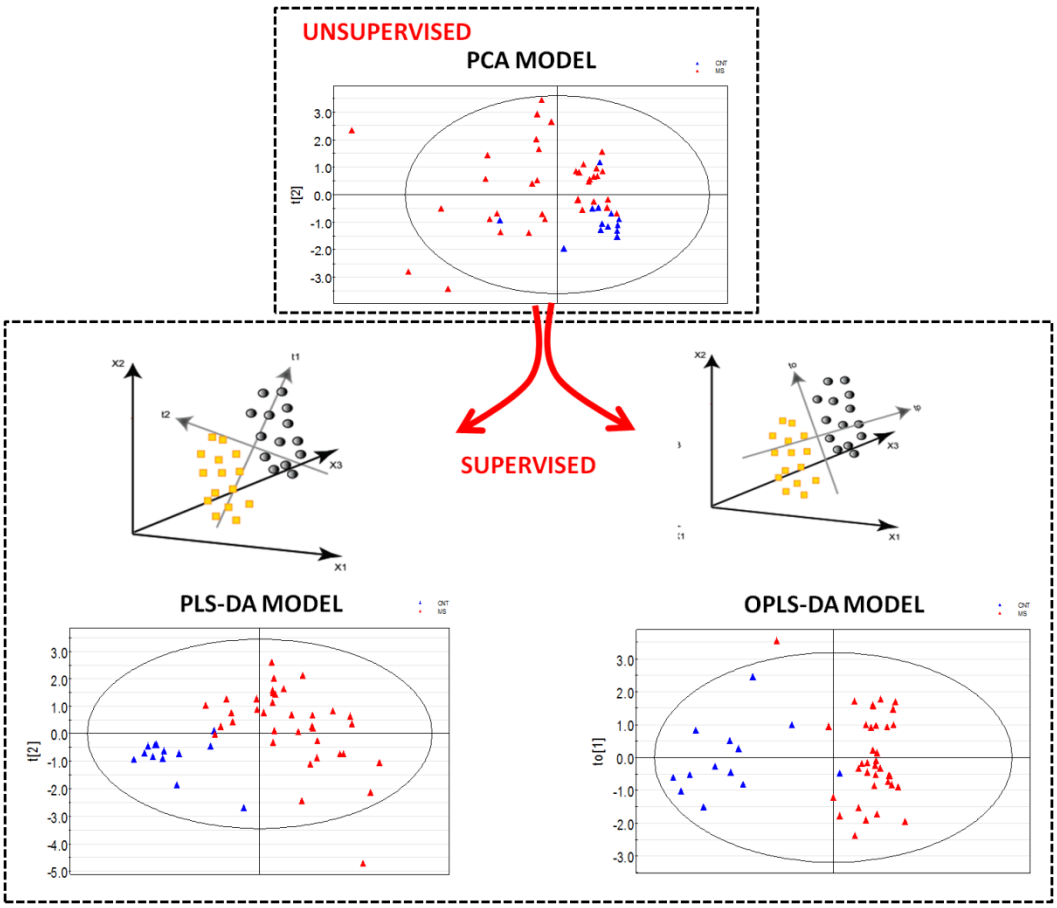


Figure 2-26: The use of multivariate unsupervised and supervised approach to investigate differences in the data set and identify biomarkers in two groups of samples (blue vs red triangles); PCA : principal component analysis, PLS-DA: partial least square discriminant analysis, OPLS-DA: orthogonal partial least square discriminant analysis. Modified from (Wiklund et al., 2007)

Partial least square (PLS-DA) regression is a supervised method that produces surrogate features (latent vectors) that explain, as much as possible, the covariance between the class belonging data (Hastie *et al.*, 2002). Orthogonal partial least square discriminant analysis (OPLS-DA) (Trygg and Wold, 2002) is a modification of PLS, which separates systematic variation in X into two parts: one part is linearly related to Y and one is orthogonal to Y (see figure 2-26). OPLS models consist of two blocks of modelled variation: (1) the Y-predictive block, which is the interclass variation and (2) the Y-orthogonal block, which is the uncorrelated variation and is therefore the intra-class variation.

Once the OPLS-DA is fit, Simca allows the identification of the important features driving the separation. The strategy for the identification of the interesting metabolites passes by

the creation and the analysis of the S-plot. These types of plot visualize the variable influence on a model. It is a scatter plot, that combines the covariance and the correlation, loading profiles resulting from the projection based model such as the OPLS-DA. It is indeed a combination of contribution (covariance, on the y axes in Figure 2-27)and reliability (correlation, on the y axes) (Wiklund *et al.*, 2007). The selection of the potentially interesting metabolites (variables) is a compromise between reliability and contribution; In the S-plot these are highlighted by circles in Figure 2-27 a. In the loading plot graph with Jack-knifed confidence intervals (another way of presenting the information), metabolites with high positive and negative contributions and with acceptable confidence intervals are recognised as important.

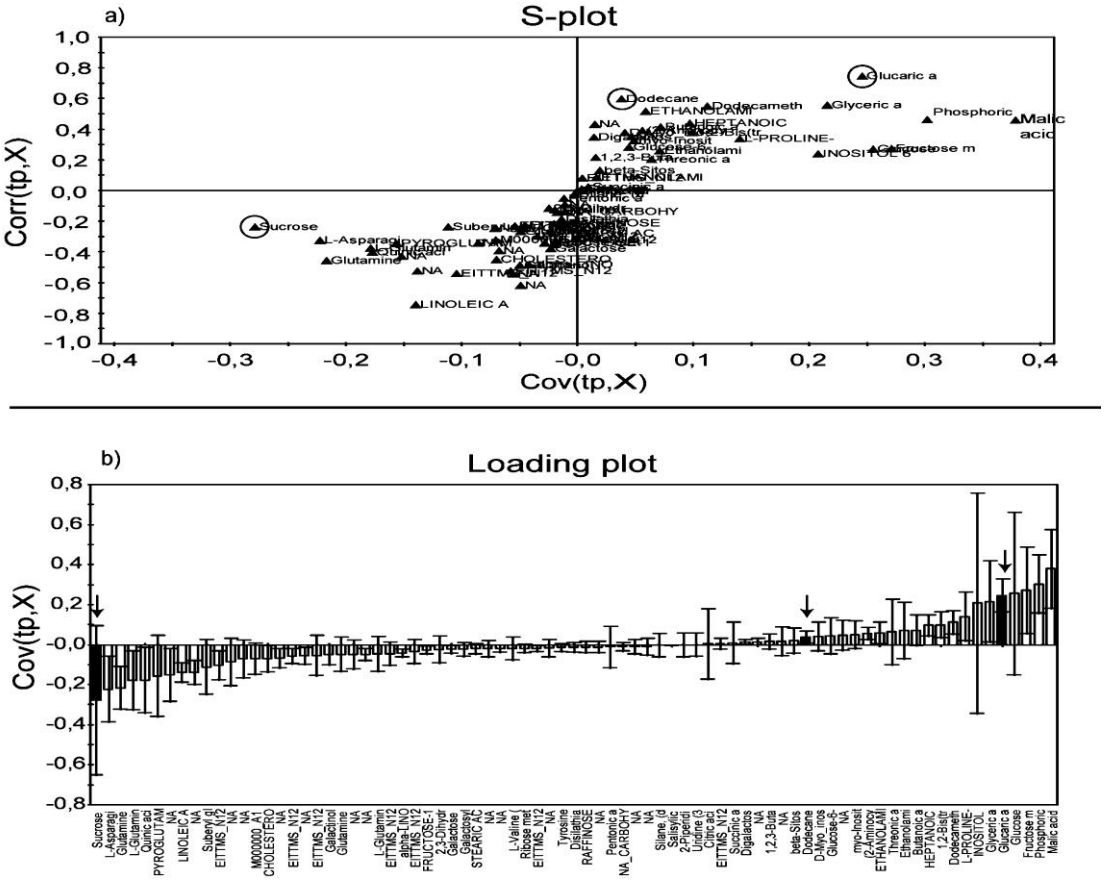


Figure 2-27: Strategy for identification of interesting metabolites for line 5. (a) S-Plot, three metabolites are highlighted by circles to demonstrate different regions in the S-plot. (b) Loading plot with jack-knifed confidence intervals. The arrows indicate the metabolites shown in (ce). (Wiklund *et al.*, 2007).

After the analysis of the important features, the analysis of data usually continues with the construction of a predictive model. The basic modelling consists of a learning phase, a test phase and an application phase (Berrar *et al.*, 2007).

The reason for producing a model out of the data, is to have generalization ability. The prediction error can be divided into bias and variance. They are complementary; the smaller the bias, the larger the variance and vice versa. An ideal model should have low variance (high precision), and low bias (high accuracy).

In Simca, the cross validation works according to the following steps:

- Parts of the data are kept out of model development.
- The kept-out parts are then predicted by the model.
- The predictions of the kept-out parts are compared with the actual values.
- 1-3 is repeated until all parts have been kept out once and only once[10].

SIMCA-P+ uses the approach of Krzanowski (Eastment and Krzanowski, 1982) where in two sub rounds, data are first kept out observation-wise (row-wise) to get a set of loading vectors, and second data are kept out variable-wise (column-wise) to get a set of score vectors.

For the cross validate predictive ability (Q^2Y) and the total explained variance (R^2X), the closer to 1 they are, the better. Usually models start to be acceptable when both Q^2Y and R^2X are > 0.5.

[10] SIMCA-P+ 12 user guide.

Chapter 3

THE IMPACT OF A RICE BASED DIET ON URINARY ARSENIC[11]

1. Introduction

Inorganic arsenic (iAs) is a non-threshold, class I carcinogenic agent (IARC, 2004), which along with other arsenic species, can be found in water and food as a result of geogenic (Nickson *et al*., 1998, Lindberg *et al*., 2006) and anthropogenic processes (Williams *et al.*, 2005b). Exposure to iAs through the dietary route and its impact on human health is of increasing concern (Meharg *et al.*, 2009c, Mondal *et al.*, 2010). Rice, a staple food for about half of the world's population, can accumulate arsenate (AsV), arsenite (AsIII) and dimethylarsinic acid (DMA)(Meharg, 2007, Zhao *et al.,* 2009). Inorganic arsenicals in food are considered more toxic than the organic forms and risk assessments in food are usually based on iAs content (NRC, 1999). The majority of the studies on human exposure to arsenic and its impact on human health have so far focused on arsenic exposure from drinking contaminated water (Haque *et al*., 2003, Agusa *et al.,* 2006, Argos *et al*., 2010)

[11] This chapter has been published as a paper in the Journal of Environmental Monitoring. It received the front cover of the month of February 2011. Figure 3-1 shows the graphical abstract associated to the paper.

with only a few on exposure from food including rice (Cleland *et al.,* 2009, Williams *et al.,* 2007, Xue *et al*., 2010, Liao *et al*., 2010). Unlike arsenic limits in drinking water, there are currently no EU, US or WHO limits for either total arsenic (As_t) or iAs in foodstuff (Francesconi, 2007).

Rice is the dominant source of dietary intake of iAs in the UK and in Europe (EFSA, 2009) and accounts for 30-60% of total iAs intake (Meharg, 2007). Baseline concentrations of As_t in white rice at a global market level range from 0.04 mg kg^{-1} (Egyptian) up to 0.28 mg kg^{-1} (French)(Meharg *et al.*, 2009c). The UK imports its rice from different countries including India (22.3%), the USA (12.6%) and Italy (12.7%) (Meharg, 2007). Indian rice imported in the UK has a mean As_t content of 0.05 mg kg^{-1}, the Italian has 0.21 mg/kg and the American has 0.26 mg kg^{-1}. Most of the arsenic in Indian and Italian rice sold on the UK market is inorganic (56% and 58% respectively), while for American rice the proportion of iAs is lower at 37% (Meharg, 2007).

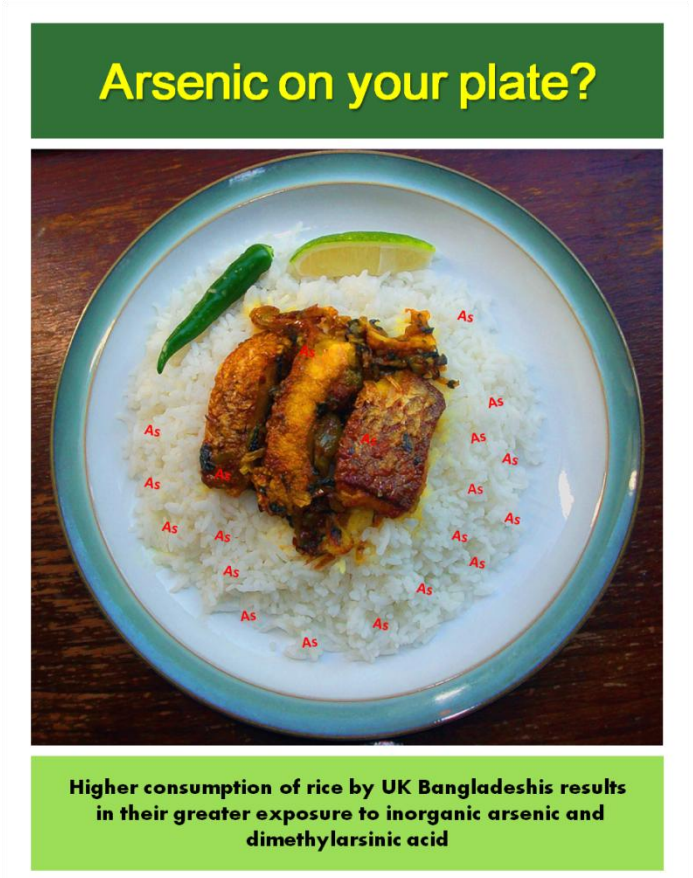


Figure 3-1: Graphical Abstract of research content of this chapter

The European Food and Safety Authority (EFSA) has estimated a 7.8 fold higher iAs daily intake for certain ethnic groups with greater rice consumption than the average European consumer (EFSA, 2009). Despite this, no comparative biomonitoring studies have been carried out on European ethnic communities to evaluate the iAs and As_t intake through rice consumption. Moreover, two previous studies have demonstrated increased urinary excretion of iAs and DMA after rice ingestion in one (Pearson *et al*., 2007) and two (He and Zheng, 2010) volunteers.

Consumption of rice *pro capite* for Bangladeshis in their country of origin is 445 g /day (d.w.) (Meharg *et al.*, 2009c), while the rice consumption decreases to 251 g/day of rice (d.w.) when they move to the UK (Meharg, 2007). Nevertheless, the Bangladeshi population still represents the largest rice consumer in the UK compared to other ethnic groups, with an average of 30 times more consumed rice than White Caucasians (Meharg, 2007). Meharg *et al.* estimated that on average, the UK Bangladeshi consumes 25 μg arsenic per day from rice, and highlighted the need for a survey on rice consumption habits of this ethnic group (Meharg, 2007). It is noteworthy that the Bangladeshis living in the UK suffer disproportionately from diabetes and cardiovascular disease compared to other groups (NHS, 2006). Despite the evidences that low exposure to iAs can lead to adverse health impacts (Fu *et al*., 2010, Ettinger *et al.,* 2009), and suggestions arising from the scientific community, no studies have investigated if the UK Bangladeshi population is exposed to a greater level of arsenic as a result of their higher rice consumption. In order to address this gap in knowledge, High Performance Liquid Chromatography Inductively Coupled Plasma Mass Spectrometry (HPLC-ICP-MS) was employed for qualitative and quantitative analysis of the main urinary arsenicals in non-occupationally exposed Bangladeshi volunteers resident in the UK. For comparison, urine biomonitoring in a group of white Caucasians living in the UK was also performed. The urinary arsenic analysis was complemented through the use of a food frequency questionnaire detecting dietary and life-style habits with particular focus on rice and fish consumption.

2. Experimental

2.1 Sample collection and study population

Spot urine samples from Bangladeshi and White Caucasian volunteers living in the UK (Leicester and London) were collected during March-September 2009 in polypropylene

tubes (Fisher Scientific, Loughborough, UK) and stored at -20º C within a few hours, till the day of the analysis. This study was approved by the Faculty of Health and Life Sciences Human Research Ethics Committee of De Montfort University and each volunteer gave an informed consent. Volunteers completed an anonymous questionnaire and kept a food diary for the two days before urine collection. Questions included demographic information, dietary habits such as rice (type, portion and frequency), fish, seafood and mushrooms intake. Volunteers were not asked to avoid eating fish and seafood in the days before collection in order to obtain a more realistic picture of the total dietary arsenic intake. Only non-smoker volunteers were included in this study, since cigarettes contain arsenic (Lindberg *et al*., 2009). However, betel quid (a mixture of Areca nut, Piper betel leaf, slaked lime and tobacco) chewers were included in this study since tobacco is not always used in the betel quid. Furthermore, to explore the possible intake of arsenic from betel quids, a comparison was made with the urinary arsenic levels in chewers and non-chewers.

Forty nine non-smoker volunteers participated in this study: 37 were Bangladeshi (mean age: 48, SD: 16) and 12 white Caucasians (mean age: 32, SD: 9). All the Bangladeshis were born in Bangladesh and abstained from drinking alcohol; the majority of the white Caucasians declared to drink from 6 to 10 units of alcohol per week. Bangladeshis drank 2.2 cups of tea and 0.2 cups of coffee per day on average, while the white Caucasians had 1.6 cups tea/day and 1.6 cups coffee/day. Almost 70% of the interviewed people in Bangladeshi community declared to drink tap water and 50% in the Caucasians.

2.2. Chemicals

Clinical screening of pH, leukocytes, nitrite, proteins, glucose, ketones, urobilinogen, bilirubin, haemoglobin and blood in urine was performed with a Combur 9 test (Roche Diagnostics Ltd, Burgess Hill, UK). Specific gravity (SG) was measured with a digital refractometer (PA/L0S, Atago co. Ltd, Tokyo, Japan).

Nitric acid (HNO_3, 69.5%, TraceSelect, Fluka Analytical, Buchs, Switzerland) and hydrogen peroxide (H_2O_2, >30% w/v, Fisher Scientific, Loughborough, UK) were used for urine digestion. Standards between 0 and 300 µg As /L were prepared in 3.3% nitric acid solution from a multi-element standard solution (10 mg /L, CLMS-2N, SPEX CertiPrep, Stanmore UK). Rh (10 µg/l) in 2 % nitric acid was used as an internal standard.

Arsenic solutions for species identification were prepared by dissolving the relevant amount of the appropriate reagent in water: DMA was prepared from cacodylic acid (dimethylarsinic acid, $C_2H_7AsO_2$) (Sigma-Aldrich, UK), MA from disodium methylarsenate ($CH_3AsNa_2O_3$) (Chem Service, USA), arsenate from sodium arsenate hydrate ($Na_2HAsO_4.7H_2O$) (Fisher Scientific Ltd, UK). Arsenobetaine (AB; $(CH_3)_3AsCH_2COOH$) was obtained from Fluka Analytical (Buchs, Switzerland).

All reagents used were of analytical or higher grade and dilutions were made with high-purity deionised water (>18 MΩ, ELGA Water Systems, UK).

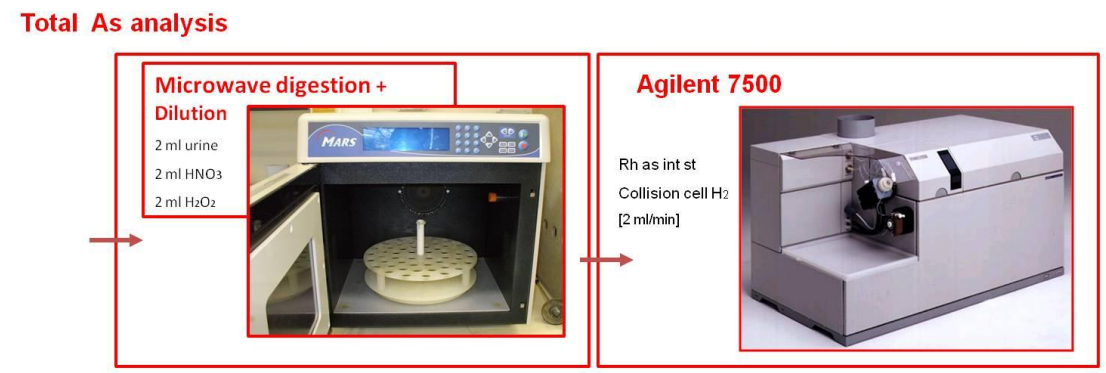
For speciation, an ammonium phosphate buffer ($NH_4H_2PO_4$) was prepared from orthophosphoric acid (H_3PO_4, 85%) and ammonia (both from AnalaR, BHD Chemicals Ltd., Poole, England). An anionic exchange column PRP-X100 (Hamilton, Reno, NV) was used. Standards for quantification of all the arsenic compounds identified in urine, were based on DMA and prepared from cacodylic acid in high-purity deionised water up to the final concentration of 20 μg As /L. The reason for doing so is that there is no significant difference between using only DMA for quantification or using mixtures of AsV, MA and DMA. In both cases there will be species present for quantification which are not included in the standard mix. ICP-MS is mostly insensitive to the molecular form of the element to be measured; therefore using only one stable As-species for quantification is justified.

Trace elements in urine (lot 0511454, Seronorm) reconstituted in ultra-pure water was used as the certified reference material.

2.3. Total arsenic analysis

After defrosting and agitating, 2 ml of urine was digested with 1:1 nitric acid and H_2O_2 in a microwave, with an accelerated reaction system (MARS, Matthew Inc. USA), up to 95ºC (power 600W, pressure at 120 PSI). After cooling, samples were diluted to 1:15 with water. Analysis was performed with an ICP-MS 7500c (Agilent Technologies, USA) equipped with a H_2 collision cell (2 mL/min). Rh 10 μg /L was added with an inline channel. The monitored signals were As (m/z 75), Se (m/z 77, 78) Rh (m/z 130), seven replicates measurements were performed per sample. A set of standards including blanks were measured every 40 samples, along with the certified reference material. Figure 3-2 A and B show a schematic representation of methods for both total and speciation analysis.

A)



B)

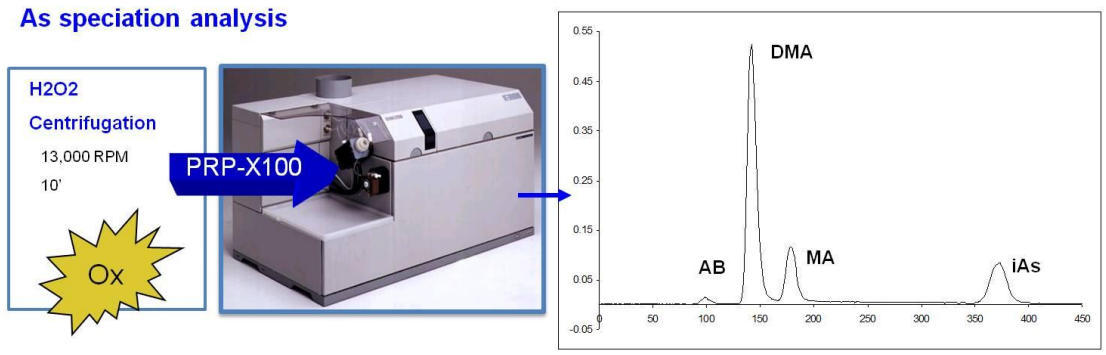


Figure 3-2: Urine preparation for total arsenic (A). A microwave digestion was needed before analysis with an Agilent 7500 ICP-MS using collision cell. (B): oxidation by mean of H_2O_2 was performed before separating arsenic species on a anionic exchange column. Chromatogram of elutes species is represented as well.

2.4. Arsenic speciation analysis

One ml of urine was oxidized with 100 µl of H_2O_2 and centrifuged at 13,000 rev min^{-1} (see Figure 3-2B). The supernatant was analyzed without further dilution for speciation analysis according to the method described by Williams *et al*. (Williams *et al.*, 2005b) , using an 1100 series HPLC system (Agilent) coupled with a 7500c series ICP-MS. An anionic exchange column was used with an ammonium phosphate (pH= 6.2) running buffer at a flow rate of 1 mL/min. A volume of 100 µL of urine was injected onto the column via an autosampler. After the column, internal standard solution (10 µg/L Rh in 2% nitric acid) was added using a peristaltic pump before detection by ICP-MS. Obtained signals were quantified based on peak area by WinFAAS 1.0 software (Kurt Kalcher, 1994).

Identification of As species was performed by the addition of Arsenobetaine (AB), dimethylarsenic Acid (DMA), monomethylarsonic acid (MA) and arsenate (AsV) to a

sample of urine; for other samples, urinary arsenicals were identified by comparison of the elution times with the spiked urine sample. A slight shift of the peaks was seen during the run, which did not affect the recognition of main arsenicals here considered. Some chromatograms are reported in Figure 3-3.

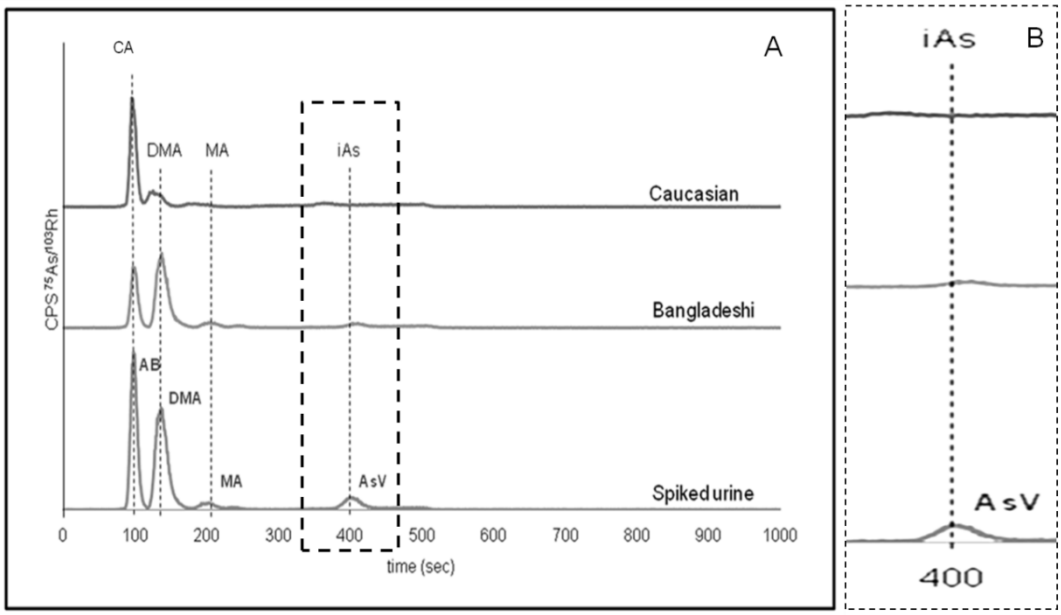


Figure 3-3: A: stacked chromatograms obtained with anionic exchange on HPLC-ICP-MS for urine. Urinary chromatograms shown represents: a UK white Caucasian (on the top) subject and a UK Bangladeshi (in the middle). Identification of As species has been performed by addition of Arsenobetaine (AB), dimethylarsenic Acid (DMA), monomethylarsonic acid (MA) and arsenate (AsV) to urine (on the bottom); note that species eluting at the same retention time of AB are indicated as cationic compounds (CA).CPS: count per second; B: a zoom in the dotted region highlighted.

3. Results

3.1. Questionnaire data

The Bangladeshi group had a much higher daily rice consumption with an average of 357±295 g/day (range: 28-1200 g/day) of cooked rice compared to white Caucasians: 17± 14 g/day (range: 3-56 g/day). In regards to the type of rice consumed, in the Bangladeshi community, 51.6% of volunteers consumed American long grain rice, while 45.2%

indicated consuming Basmati rice. Within the Bangladeshi group, 29.4% had rice once a day and 67.6% twice a day.

Average body mass index (BMI) was 26.4 ± 9.4 and 21.1 ± 7.8 for the Bangladeshis and white Caucasians, respectively. The majority of the Bangladeshi and White Caucasians had fish in the last two days before urine collection.

3.2. Total arsenic in urine

Certified reference material was prepared and analysed in 4 replicates along the run and showing an average recovery of 110% (measured value: 156.9±7.2 µg/L, certified values: 142±6 µg /L and reference range: 130 – 154 µg /L) for As; the limit of detection (LOD) calculated as (3*SD blanks/slope)*average dilution factor was 0.032 µg /L and the limit of quantification (LOQ) calculated as (10*SD blanks/slope)*average dilution factor was 0.951 µg /L. None of the analysed urine samples was below the limit of detection. Table A1-1 in the Appendix reports the average and error (for the 7 readings) for the analysed urine samples. Relative standard deviation of replicates of one urine sample was 1.7%.

In order to adjust for individual variation in spot urine concentration either urinary creatinine (Brima *et al.*, 2006b), (Kile *et al.,* 2009) or specific gravity (Nermell *et al.*, 2008b) (SG) is generally used. Creatinine has been reported to be significantly associated with urinary arsenic in a rural population of Bangladesh by Nermell *et al.* (Nermell *et al.*, 2008b) and to vary with body weight and fat free mass (Baxmann *et al*., 2008) and dietary protein intake (Mayersohn *et al.,* 1983). Nermell *et al.* (Nermell *et al.*, 2008b) concluded that specific gravity may also be influenced by age, gender, and body size, but the influence is less than for creatinine. Therefore, in this study SG was chosen as the preferred method to adjust for variation in urine concentration. SG values for both the Bangladeshis and the Caucasians had means of 1.014 (SD 0.006) and 1.020 (SD 0.005), respectively. It was normally distributed in both groups and t-test analysis showed a significant difference ($p<0.001$). To correct for SG, the following equation (Nermell *et al.*, 2008b) was used:

U-As SG [µg/L] = (Urinary Arsenic [µg/L] x (1.012-1)/(Specific Gravity - 1.000) (Equation 5)

Table 3-1 A shows urinary As_t with SG adjustment (U-As SG); values without SG adjustment (U-As) are available in Table 3-1 B. As_t was not normally distributed and positively skewed. The geometric mean of U-As SG was 28.7 µg /L for the Bangladeshis and 22.0 µg /L for the white Caucasians. A Mann-Whitney test showed no significant difference in As_t, with/without SG correction in relation to ethnicity and gender.

3.3. Urinary As speciation

As a result of the oxidation by H_2O_2 step, all the trivalent arsenic species in urine (including methylated trivalent arsenicals and dimethylarsinothioic acid) eluted as the corresponding pentavalent oxo-forms on the anionic exchange column. Arsenic species were identified by retention time comparison of a mixture of arsenobetaine (98 sec), DMA (141 sec), MA (217 sec) and AsV (407 sec) in water and in real urine as shown in Figure 3-3. Recovery from urine reference material was 109%. Chromatographic recovery was 90 +/-19 %. Since a cationic exchange column was not used to separate arsenocholine (AC), trimethylarsine oxide (TMAO) and the tetramethylarsonium ion (TETRA), these compounds, if present, eluted at the front of the chromatogram together with AB. However, considering that none of the volunteers declared to have eaten seaweed in the two days before collection, we assume that AB is the main compound eluting in the front peak, with a possible small contribution to the signal from AC. Hence, these compounds will be addressed as 'cationic arsenic'(CA) in this thesis.

The LOD was (3 SD blank/slope) 0.005 µg /L and LOQ (10 SD blank/slope) was 0.015 µg/L. For statistical analysis, concentration zero values and concentrations below LOQ, were substituted with 0.007 µg /L.

Urinary species of Caucasians and Bangladeshis are statistically described and compared (Mann-Whitney test) in Table 3-1 A after SG correction (U-As SG), data without SG correction (U-As) in Table 3-1 B; Figure 3-4 represents urinary arsenic species in the two ethnic groups versus the sum of all the species detected by HPLC-ICP-MS. Urinary DMA was 3-fold higher in the Bangladeshis compared to the white Caucasians without and with SG correction. In contrast, cationic arsenic was much higher in the white Caucasian group without and with SG correction.

No significant difference was found for MA between the two groups. The sum of DMA, MA and iAs medians for the Bangladeshis is about 3-fold higher (18.3 µg /L) compared to the

Caucasians (5.5 µg /L). In both ethnic groups, DMA accounted for more than 90% of the three main species that are normally considered for arsenic exposure and risk assessment (MA, DMA and iAs). There was no difference in relation to gender for any of the analysed species in the whole study population.

Table 3-1 Descriptive statistics and non parametric analysis of urinary arsenicals for Bangladeshi (Bangl) and Caucasians (Cauc) before (A) and after (B) specific gravity correction; Ast total arsenic; CA: cationic arsenic; DMA: dimethylarsinic acid; MA: monomethylarsonic acid; iAs: inorganic arsenic.

A [µg/L]	As_t		CA		DMA		MA		iAs	
	Bang	Cauc	Bang	Cauc	Bang	Cauc	Bang	Cauc	Bang	Cauc
Mean	43.7	78.0	13.2	71.4	22.5	8.47	0.610	0.250	0.850	0.400
DS	46.4	99.2	30.9	98.0	22.6	8.47	0.920	0.460	0.710	0.320
Geoemean	28.7	36.6	3.51	27.6	15.3	5.66	0.120	0.0400	0.390	0.180
Median	26.5	30.8	2.61	21.7	17.2	5.11	0.280	<LOD	0.820	0.370
Min	4.55	5.15	0.470	1.77	2.17	1.93	<LOD	<LOD	<LOD	<LOD
25th %	17.5	14.3	1.04	11.03	8.07	2.48	<LOD	<LOD	0.350	0.110
75th %	45.5	81.0	6.96	85.6	27.5	10.0	0.830	0.380	1.05	0.690
Max	124	286	157	271	123	28.9	4.20	1.59	3.20	0.870
P (95% CI)	0.634		***<0.001***		***0.004***		0.117		***0.027***	

B [µg/L]	As_t		CA		DMA		MA		iAs	
	Bang	Cauc	Bang	Cauc	Bang	Cauc	Bang	Cauc	Bang	Cauc
Mean	35.7	51.7	9.83	49.2	19.3	5.15	0.600	0.140	0.750	0.240
DS	28.0	75.2	20.6	75.1	14.6	5.32	0.740	0.230	0.520	0.210
Geoemean	28.7	22.0	3.51	16.6	15.2	3.40	0.0500	0.0300	0.390	0.120
Median	28.4	20.6	2.93	14.9	16.9	3.16	0.410	<LOD	0.630	0.250
Min	8.3	2.38	0.600	0.820	4.62	0.920	<LOD	<LOD	<LOD	<LOD
25th %	18.8	8.12	1.28	6.08	8.87	1.61	<LOD	<LOD	0.460	0.050
75th %	36.8	51.1	5.83	50.3	25.3	6.33	0.880	0.190	0.810	0.370
Max	124	249	94.9	245	69.9	18.9	2.80	1.59	2.18	0.680
P (95% CI)	0.423		***0.002***		***<0.001***		0.174		***<0.001***	

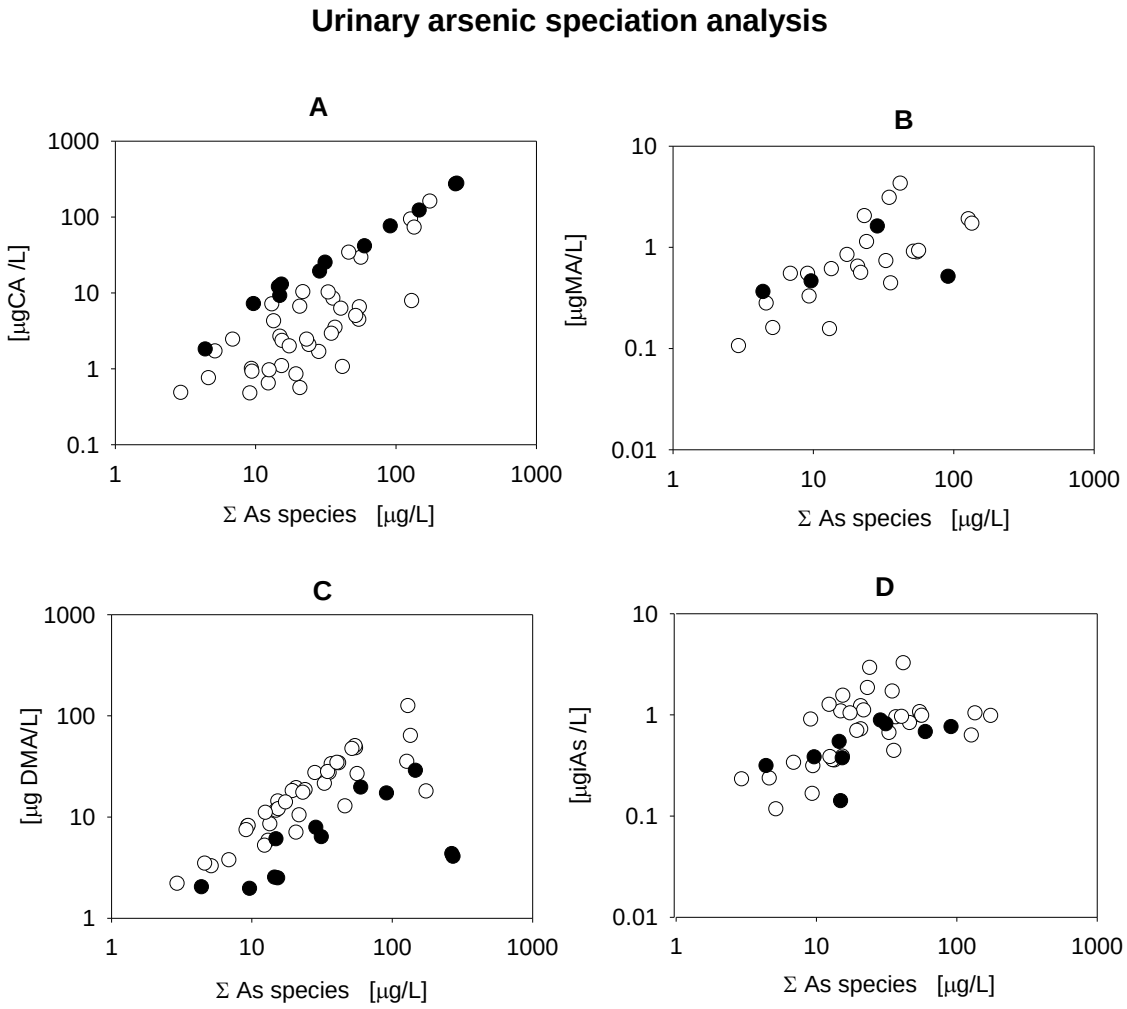


Figure 3-4: Scatter plots of species detected in urine of UK white Caucasians (black points) and UK Bangladeshis (white points). Concentrations are on a log scale, species considered are: cationic arsenic (CA) (A); monomethylarsonic acid (MA) (B), 23 samples below the LOD were excluded from the graph; dimethylarsinic acid (DMA) (C); inorganic arsenic (iAs) (D), 6 samples below LOD were excluded from the graph.

Within the Bangladeshi group, there was no appreciable difference related to gender or age for total and all the analysed arsenic species without/with SG correction. Betel quid chewing was prevalent amongst 24.3% of the Bangladeshis. However, there was no significant difference (p=0.497) in the urinary arsenic species as a sum of DMA, MA and iAs between chewers (median 21.0 μg /L; range: 3.9-65.3 μg /L) and non-chewers (median 17.1 μg /L; range 2.5-123.5 μg /L), or in the As_t (p= 0.302). Twelve out of the 37 Bangladeshi volunteers reported as being diabetic. A comparison of As_t, DMA, MA, iAs, first

methylation step efficiency (MA/iAs) and second methylation step efficiency (DMA/MA) performed between healthy and diabetic Bangladeshi did not reveal significant differences.

The questionnaire analysis revealed that the Bangladeshi group consumed 21-fold more rice than Caucasians. Most Bangladeshis (51.6%) consumed American long grain rice, which has a higher content of both iAs and DMA compared to Basmati rice sold in UK (Meharg, 2007). Figure 3-5 shows plots of the quantity of rice eaten per day (in g/day on a log scale, in the whole sampling population) versus urinary DMA and iAs content. An increase in urinary DMA levels is seen with the increase of daily intake of rice and rice frequency consumption as shown also in Tables 3-2 and 3-3.

Table 3-2 Linear regression between urinary DMA and iAs and daily rice consumption

x	y	P
DMA	Rice consumption	0.025
DMA	Log(Rice consumption)	0.054
Log iAs	Log(Rice consumption)	0.012
iAs	DMA	0.002
Log iAs	Log DMA	0.010

Table 3-3 Effect of rice frequency consumption on urinary arsenic species in UK Bangladeshi[a]

x	n	%*	DMA'	MA'	iAs'
Rice once a day	10	29%	8.3	0.1	0.8
Rice twice a day	23	68%	18.1	0.2	0.5
P (95% CI)			0.016	0.743	0.180

[a] It shows the difference in urinary arsenicals between volunteers eating rice daily once a day and twice a day within the Bangladeshi group who answered the question on daily rice consumption (n=33); (*) percentage calculated on number of volunteers; (') geometric mean.

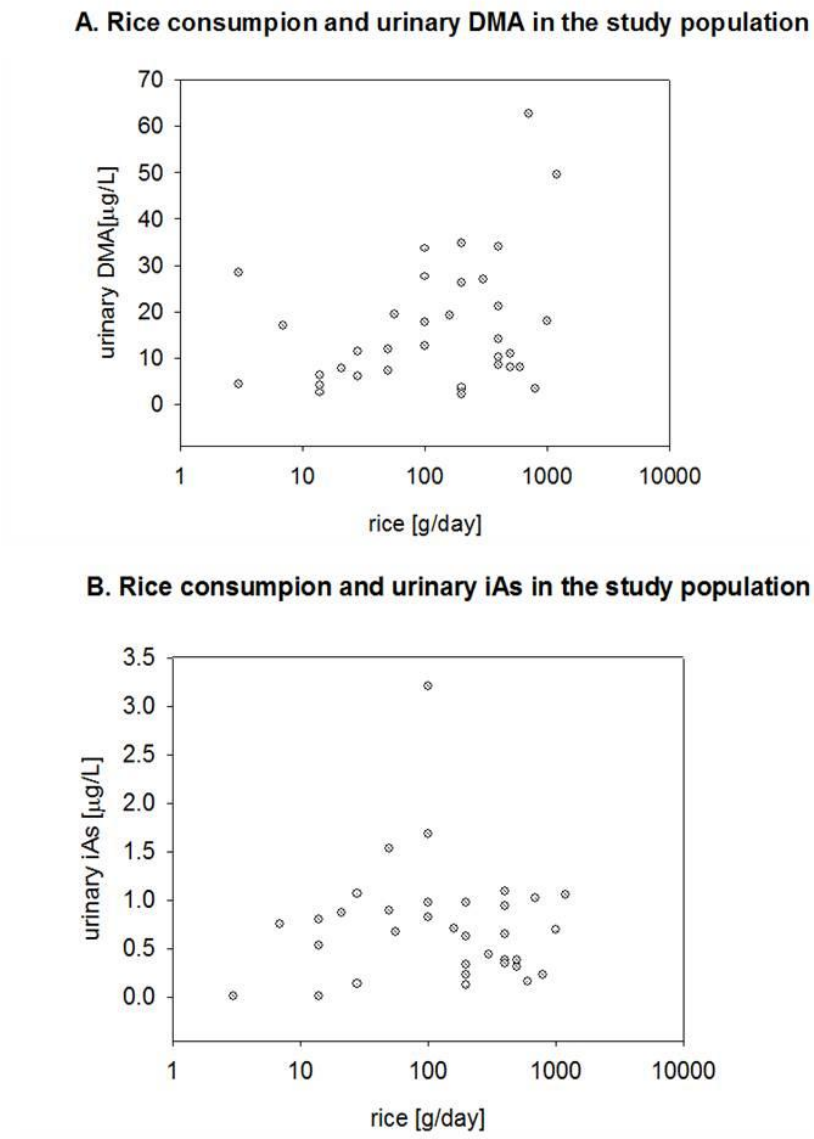


Figure 3-5: Scatter plots showing the relation between daily rice consumption (on a log scale) and urinary DMA(A) and iAs (B) in the study population including both UK Bangladeshi and white Caucasians who provided information on rice intake.

4. Discussion

This is the first study to monitor arsenic exposure in Bangladeshis living outside of Bangladesh. Bangladeshis are proud of their diet that has generally consisted of rice and fish and often refer to themselves as "*Mache-Bhate-Bangali"* which can be translated as *"Rice and Fish Makes a Bengali"*. With the exception of some differences in quantities of rice and other foods consumed, the UK Bangladeshi community is known to maintain a

similar diet to their country of origin (Nunez-De La Mora *et al*., 2007). All the UK Bangladeshi volunteers involved in this study consumed rice on a daily basis. In terms of arsenic exposure, the most relevant difference between Bangladeshi community in the UK and in Bangladesh lies is the absence of high arsenic exposure through drinking water since the British tap water meets the terms of 98/83/EC directive according to which arsenic levels should be < 10 μg /L. Although the UK Bangladeshis are not affected by As in drinking water, they show an approximately 3-fold increase in the sum of the medians of urinary iAs, DMA and MA compared to the white Caucasians. Urinary DMA, which is significantly increased in the Bangladeshi group, accounts for the greatest part of this difference. Moreover, a significant increase in urinary iAs for Bangladeshis in comparison to white Caucasians is also seen. At the same time, in this study we report substantially (21-fold) higher rice consumption for the UK Bangladeshis in comparison to white Caucasians.

This higher rice intake is the most plausible explanation for the increase in urinary DMA in the UK Bangladeshi group. Volunteers were not asked to refrain from eating seafood before the day of urine collection but a two days food diary was obtained before urine sampling. Since volunteers did not refrain from eating fish, this could potentially represent a confounding factor preventing one from attributing the arsenic levels in urine solely to rice consumption (Sirot et al., 2009). However, CA and DMA in urine of Caucasians and Bangladeshis show an opposite trend: CA is significantly higher in urine of Caucasians whereas DMA is significantly higher in the Bangladeshis. The questionnaire explains this trend since the Caucasian group reported eating sea fish and Bangladeshis consumed mainly fresh water fish. The Bangladeshi volunteers mainly reported names of imported Bangladeshi freshwater fish in the completed questionnaire. The Bangladeshi community residing in the UK originate mainly from the North-Eastern district of Sylhet (de Brito-Ashurst *et al.,* 2009) which is far from the coast and they are known to mainly consume freshwater fish. In general, freshwater fish contain less total arsenic than seawater fish (Woolson, 1983). Taken in conjunction with the fact that the Bangladeshi volunteers consume ~21-fold greater rice compared to the Caucasian group, the arsenic speciation profile of the Bangladeshi group is likely to be dominated by the known presence of DMA and inorganic arsenic in rice. Nevertheless, we cannot rule out the possibility that some of the urinary DMA and inorganic arsenic detected in the Bangladeshi group comes from fish consumption. Speciation analysis of the imported Bangladeshi freshwater fish consumed

by these Bangladeshi volunteers has not been reported as yet. However, it has been previously reported that varying quantities of DMA and inorganic arsenic is present in freshwater fish from Europe (Ciardullo *et al*., 2010), North America (Zheng and Hintelmann, 2004) and Asia (Jankong *et al*., 2007). There is no uniform distribution of arsenic species in freshwater fish and the proportion of AB and DMA levels have been found to vary depending on the type of fish analysed, from very small to significant levels (Ciardullo *et al*., 2010; Zheng and Hintelmann, 2004; Jankong *et al.,* 2007). Considering the diverse types of freshwater fish consumed by the Bangladeshis, it is possible a contribution to the urinary arsenic profile of this community does arise from this source. For a population not known to eat seaweed, it is unlikely to find breakdown products from arsenosugars in measurable quantities. Nevertheless, future studies on speciation of Bangladeshi fish and other foods are necessary.

Our hypothesis regarding rice, rather than fish, being the main source of urinary DMA in the Bangladeshi group is supported by the study of Pearson *et al.* (Pearson *et al*., 2007), who demonstrated an increase in both iAs and DMA urinary levels (up to 25.90 µg /L) in a single volunteer upon ingestion of 250 g American long grain rice. This 3.5-fold increase in urinary DMA after rice ingestion is compatible with the increase seen in the UK Bangladeshi group in comparison to white Caucasians. Urinary DMA increase after rice ingestion has been confirmed from a second study involving the use of two volunteers (He and Zheng, 2010). Our study is the first to relate urinary arsenic levels with rice consumption through the use of 49 volunteers. In light of the cited studies, the increase in urinary iAs and DMA in the Bangladeshi population living in the UK in comparison to the white Caucasian control group, is likely to be primarily due to their higher rice intake.

Urinary DMA level of white Caucasians from this study (U-As geomean: 5.7 µg /L **±** 2.5 µg /L) is, on the whole, in line with values reported for other non exposed groups in the UK (median: 3.4 µg /L) (Morton and Mason, 2006), USA (median: 3.27 µg /L for non-Hispanic white, 4.72 µg /L for Mexican American and 4.27 µg /L Non Hispanic Black) (Caldwell *et al.,* 2009) and Germany (median: 2.6 µg /L) (Heitland and Kolster, 2008) and most importantly compatible with non exposed groups in UK who consumed fish 24 hours prior to urine collection (geomean: 5.6 µg /L) (Morton and Mason, 2006) (see Figure 3-6 A).

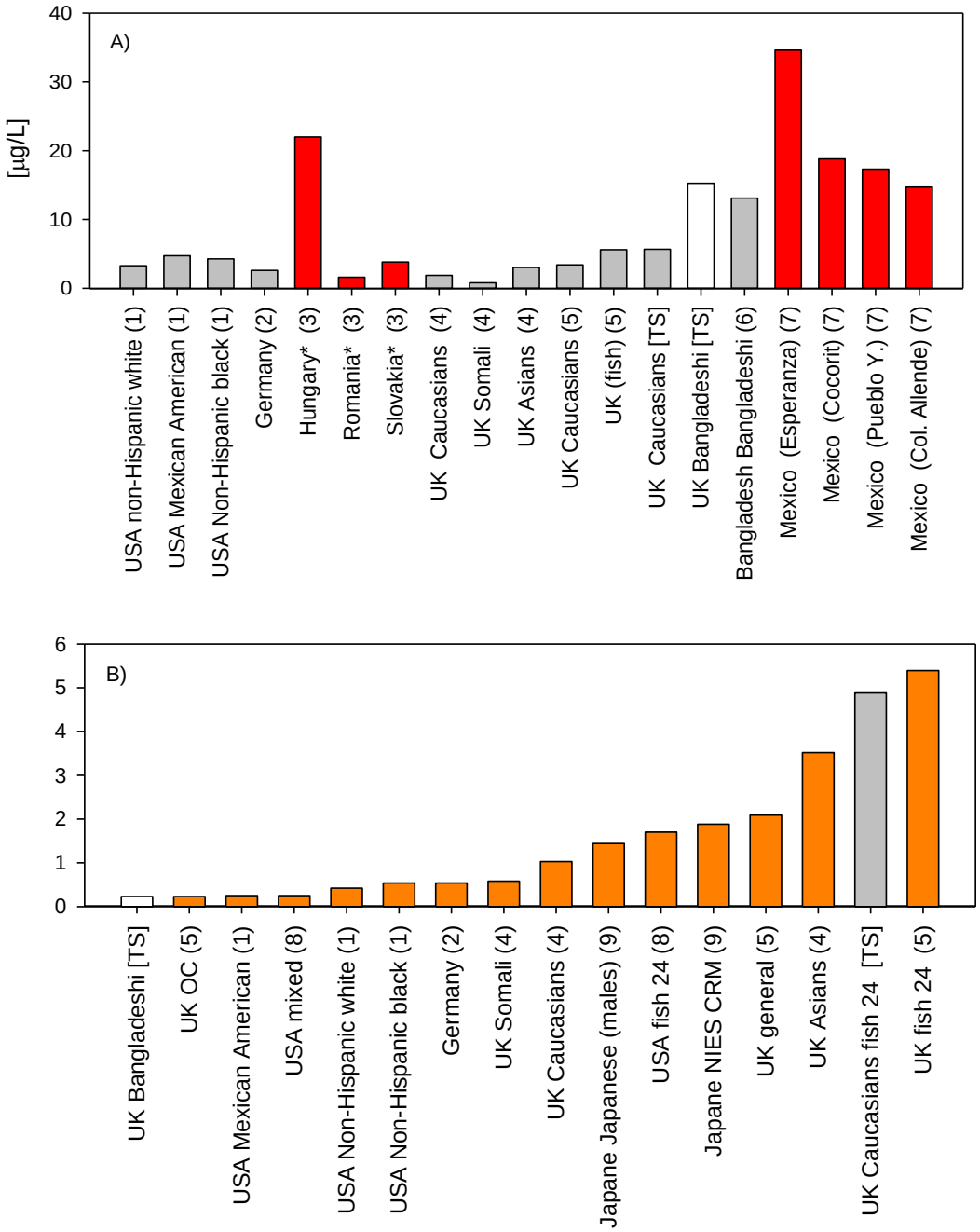


Figure 3-6: (A) DMA urinary levels from different studies. Bar charts are based on geometric mean or median (*) values: grey bars are for general population not exposed to As through drinking water (As < 10 µg/L); red bars indicate groups exposed to low As exposure levels in drinking water (10 µg/L < As < 100 µg /L). White bar indicates DMA levels for Bangladeshis living in the UK from this study (TS). (B) Comparison of relative ratios of CA/DMA: for UK Bangladeshi (white bar) and white Caucasians (light bars) from this study (TS); ethnicity when known and fish consumption in the 24 h before urine collection (fish 24) are indicated; occupationally exposed (OC) volunteers are highlighted. References: (1) (Caldwell *et al.*, 2009); (2) (Heitland and Kolster, 2008); (3) (Lindberg *et al.*, 2006); (4): (Brima *et al.*, 2006b); (5) (Morton and Mason, 2006); (6) (Kile *et al.*, 2009); (7) (Meza *et al.*, 2004); (8): (Navas-Acien *et al.*, 2010); (9): (Hata *et al.*, 2007).

In contrast, the Bangladeshi group reported in this study has the highest level of DMA and iAs in urine among the different ethnic groups (Asians, Somali and Caucasians) from Leicester (Brima *et al.*, 2006b) and in general amongst any non-exposed groups reported thus far in the UK (Morton and Mason, 2006). Figure 3-7 shows a comparison based on geometric mean of urinary arsenicals of UK Bangladeshi group from this study and other ethnic groups previously reported in Leicester by Brima *et al.* (2006b). All the volunteers in the latter and the current study were not occupationally exposed. UK Bangladeshis reported in the present study have the highest DMA% (79%) compared to Asians (22%), Somali (59%) and Caucasians (48%) in Leicester.

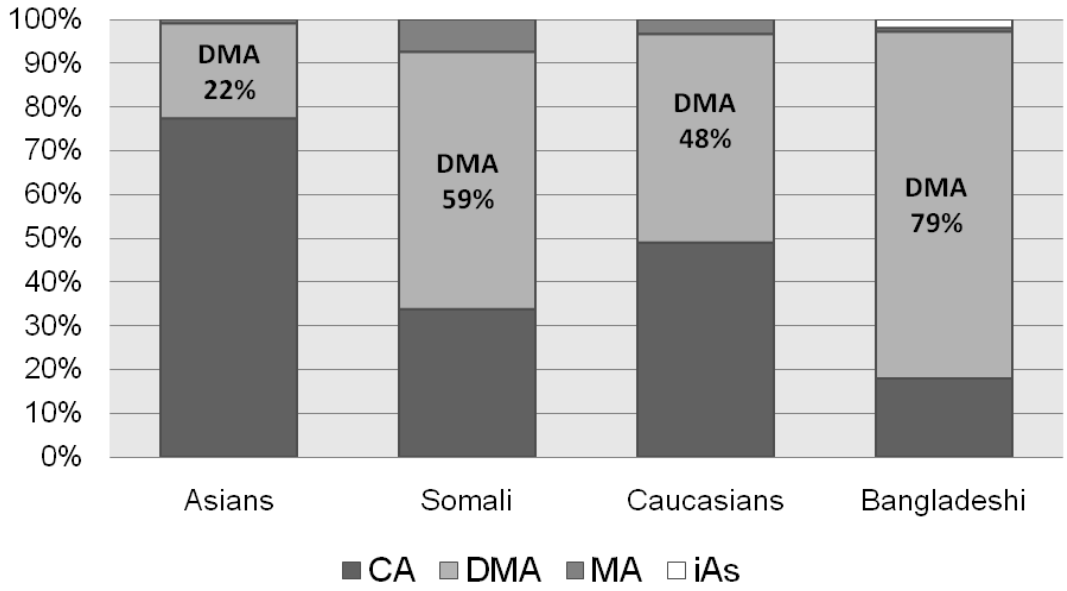


Figure 3.7: Urinary arsenicals in different ethnic groups in the UK: comparison of relative proportion of urinary arsenicals found in UK Bangladeshi population (from this study) with other Asians (n=21), Somali (n=22) and Caucasians (n=20) living in the UK based on geometric mean reported by Brima *et al.* (2006b).

Table 3-4 presents an international comparison of urinary arsenic from different populations around the world: DW is used when volunteers drink water with As> 10 µg/L, OC for occupationally exposed volunteers and G for non-exposed groups (populations that are not exposed to high levels of arsenic from any anthropic or natural sources). In figure 3-6A, a comparison of urinary DMA levels for UK Bangladeshis , general population and low-As drinking water exposed population groups is shown. Volunteers drinking water with 10 µg /L< As < 100 µg /L are here considered as low-As drinking water exposed

Table 3-4: International comparison of urinary arsenic species

Country (city/district)	Ethnicity	type	n	tot As	CA	DMA	MA	iAs	Ref
USA	*Non-Hispanic white*	G	1074107410	7.12	1.37	3.27	-	-	(Caldwell *et al.*, 2009)
	Mexican American	G		9.29	1.19	4.72	-	-	(Caldwell *et al.*, 2009)
	Non-Hispanic black	G		11.6	2.29	4.27	-	-	(Caldwell *et al.*, 2009)
Germany (Bremen)	-	G	82	7.9	1.4	2.6	0.33	0.26	(Heitland and Kolster, 2008)
Hungary (Csongrad)	*Caucasians*	DW	61	*32	-	*22	*5	*3.5	(Lindberg *et al.*, 2006)
Romania (Bihor)	*Caucasians*	DW	56	*2.1	-	*1.6	*0.27	*0.23	(Lindberg *et al.*, 2006)
Slovakia (Nitra)	*Caucasians*	DW	69	*5	-	*3.8	*0.68	*0.29	(Lindberg *et al.*, 2006)
UK	*Caucasians*	G	20	13.74	1.92	1.86	0.13		(Brima *et al.*, 2006b)
	Somali	G	22	6.92	0.46	0.8	0.1	<LD	(Brima *et al.*, 2006b)
	Asians	G	21	27.3	10.67	3.03	0.1	<LD	(Brima *et al.*, 2006b)
UK	*Caucasians*	G	34		7.1	3.4	0.1	3.1[$]	(Morton and Mason, 2006)
	Caucasians	F	31		30.2	5.6	0.1	3.3[$]	(Morton and Mason, 2006)
	-	OC	49		11.3	48.9	23.5	23.3	(Morton and Mason, 2006)
UK	*Caucasians*	G	12	36.3	27.6	5.66	0.04	0.180	TS
	Bangladeshi	G	37	28.7	3.5	15.3	0.120	0.390	TS
India (Mamudpur)	-	DW	27	[a]243.3	-	[a]136.1	[a]25.5	[a]83.3	(Tokunaga *et al.*, 2005)
India (Skrikrishnapur)	-	DW	52	[a]144.7	-	[a]104.3	[a]15.5	[a]24.9	(Tokunaga *et al.*, 2005)
India (Bakshipur)	-	G	9	[a]36.7	-	[a]26.1	[a]6.4	[a]4.2	(Tokunaga *et al.*, 2005)
Bangladesh (Pabna)	*Bangladeshi*	DW	2971	32.3	-	20.4	2.1	3	(Kile *et al.*, 2009)
	Bangladeshi	G	1111	16.7	-	13.1	1.3	1.8d	(Kile *et al.*, 2009)
Taiwan	-	DW	1189	-	-	[a]60.8	[a]9.21	[a]4.85	(Huang *et al.*, 2009)
	-	G	205	-	-	[a]52.55	[a]3.21	[a]1.42	(Huang *et al.*, 2009)

Country (city/district)	Ethnicity	type	n	tot As	CA	DMA	MA	iAs	Ref
Japan	*Japanese*	G	210	141.3	61.3	42.6	3.1	3.6	(Hata *et al.*, 2007)
Mexico	-	DW	14	64.5	-	34.6	6.3	15.7	(Meza *et al.*, 2004)
(Esperanza)									
(Cocorit)	-	DW	10	29.5	-	18.8	4.2	4.6	(Meza *et al.*, 2004)
(Pueblo Yaqui)	-	DW	9	38.4	-	17.3	2.9	7.9	(Meza *et al.*, 2004)
(Col. Allende)	-	DW	10	36.2	-	14.7	2.3	7.0	(Meza *et al.*, 2004)

All the values are reported as µg /L; (a): arithmetic mean. (*): median, if not indicated geometric mean; (<LD): below limit of detection; ($) these values are for smokers and no iAs was detected in non-smokers. Population groups are categorised as exposed through drinking water (DW) when a level >10 µg/L is reported, occupationally exposed (OC), general with fish consumption reported in 24-48 h before collection (F), and general (G). (TS: this study)

groups. The UK Bangladeshi group clearly shows the highest level of DMA among the non-exposed volunteers. DMA levels detected are similar to the low exposure group. It is noteworthy that the urinary DMA geometric mean for the UK Bangladeshi group (15.3 µg /L) is very similar to a Bangladeshi group (13.1 µg /L) living in Pabna (Kile *et al.,* 2009) (Bangladesh) that was not exposed to As through drinking water. The authors of the latter study suggest that when drinking water is not a major contributor to the arsenic intake, diet is the main source of As exposure. However, the Bangladeshi population from Pabna reported by Kile *et al.* (Kile *et al*., 2009) shows a 4.6 and 10.5 fold increase in iAs and MA respectively compared to UK Bangladeshis. The reasons for this difference could be related to: i) higher rice consumption for residents in Bangladesh (1568 ± 524 g) (Zablotska *et al*., 2008) than in the UK (357±295 g/day); ii) the type of rice consumed is different: Bangladeshi rice contains a higher percentage of iAs (60%, iAs, average: 0.07 mg kg^{-1}) (Meharg *et al.*, 2009c) compared to American long grain rice (37%, iAs average: 0.09 mg kg^{-1}) and Indian/Pakistani Basmati (56%, iAs average: 0.03 mg/kg) rice normally consumed by UK Bangladeshis (Meharg, 2007); and finally, iii) the increase in the level of iAs and MA reported by Kile *et al*. (Kile *et al*., 2009) could be derived from the consumption of other foods such as locally grown vegetables irrigated with As contaminated water.

Fig.3-6B (derived from Table A3-2 in the Appendix) compares the CA/DMA ratio in the study population from this study with some reported groups from the literature. The Bangladeshi group in the present study has the lowest CA/DMA ratio (Figure3-6 b). In contrast, the UK white Caucasians from this study have high ratio due to their higher CA intake from seafood consumption. This supports the idea that DMA increase in UK Bangladeshi is unlikely to originate from fish consumption although this cannot be ruled out entirely as discussed earlier.

On the basis of the average quantities of rice consumed, derived from our questionnaire, a Bangladeshi consumer (0.360 kg rice/day) will ingest 32.4 µg iAs/day from American long grain rice and 10.8 µg iAs/day from Basmati rice. In contrast, white Caucasians are likely to ingest 1.53 and 0.51 µg iAs /day from American long grain and Basmati rice, respectively. This estimation for iAs intake for UK Bangladeshis (32.4 µg iAs/day) is in line with what was previously estimated by Meharg *et al*. (25 µg iAs/day) (Meharg, 2007). If the WHO limit of 10 µg /L is considered, a daily intake of 2 L of drinking water per day will result in the ingestion of 20 µg iAs/day. Therefore, a UK Bangladeshi consuming 0.360

kg /day of American long grain rice, exceeds this limit. Scatter plots (Figure. 3-5-A and B) of daily rice intake per day and urinary DMA and iAs suggest a linear relationship. A significant correlation is seen with linear regression between daily intake of rice and urinary arsenic (see Table 3-2), although the R squared values are quite low (0.1-0.2). However, our study was not a mass balance and that rice portions assessed by questionnaires are prone to error. Moreover, considering two rice consumption categories in the Bangladeshi group (Table 3-3), urinary DMA is higher (p=0.014) in volunteers consuming rice twice a day rather than once. No difference was found for MA (p=0.743) and iAs (p=0.180).

Possible factors contributing along with rice consumption to the increase of DMA in urine of Bangladeshi volunteers are: i) seaweed consumption (Feldmann *et al.,* 2000) - excluded *a priori* since none of the interviewed people reported consuming seaweed; ii) presence of arsenic species in fresh water fish that can be converted to DMA - as already mentioned we cannot exclude this possibility until speciation data on Bangladeshi fresh water fish are available.

The observed 3-fold increase in UK Bangladeshis urinary DMA in comparison to UK white Caucasians is most likely due to higher rice consumption as argued below:

I. 21-fold higher consumption of rice in Bangladeshis and the fact rice consumed is known to contain significant levels of DMA (Meharg, 2007),

II. the inverse CA/DMA trend between the UK Bangladeshis and Caucasians suggesting that consumption of AB-containing fish is far less in the Bangladeshis.

III. the compatibility of the increase in urinary DMA levels with previous studies on urinary arsenic after rice consumption (Pearson *et al.,* 2007; He and Zheng, 2010).

IV. the actual content of DMA in urine of Bangladeshis compared with all the other UK groups reported so far.

V. the similarity of UK Bangladeshi urinary DMA levels with Bangladeshis from Pabna (Kile *et al.,* 2009) unexposed to As through drinking water, with higher rice consumption than in the UK.

If the increase in urinary DMA is the result of higher DMA intake through rice, this represents a confounding factor in the estimation of risk and in the use of DMA/MA ratio to assess the methylation capacity in humans. In fact, variation in DMA intake as a result of differences in the quantities and type of rice consumed can significantly alter the DMA/MA ratio resulting in misleading conclusions. This possible confounding influence of DMA from rice intake in the evaluation of arsenic methylation in populations exposed to iAs in drinking water has not been previously addressed. This issue needs to be taken into consideration in much the same way as arsenic intake from seafood is currently considered an important factor in any risk assessment studies. In populations with high consumption of DMA rich rice, differences in DMA/MA could reflect differences in rice consumption, which can vary with gender and age. Hence, studies reporting differences in methylation capacities for such groups needs to consider the contribution of DMA from rice consumption before any definitive conclusions can be reached in the link between genetic polymorphism, nutrition, iAs exposure etc to differences in DMA/MA ratio.

5. Conclusions

HPLC-ICP-MS data presented in this study clearly demonstrates that the Bangladeshi ethnic group has statistically significant higher levels of iAs and DMA compared to white Caucasians, and this correlates well with the 21-fold higher rice consumption in the Bangladeshi community. Although the population size of this study is relatively small, the statistically significant higher iAs and DMA levels in urine of the 37 Bangladeshi volunteers is consistent with the increased urinary excretion of these species after rice ingestion shown in previous studies conducted with only one (Pearson *et al.,* 2007) and two (He and Zheng, 2010) volunteers. The UK Bangladeshis have a diet that makes them vulnerable to higher exposure to arsenic due to consumption of foods with high As levels including rice, some types of vegetables (Al Rmalli *et al*., 2005) imported from Bangladesh and possibly fish. In light of the findings presented in this chapter and of the recent demonstration that arsenic intake increases mortality for chronic diseases in Bangladesh (Argos *et al.*), a large epidemiological study could be undertaken to determine if any correlation exists between the known high mortality for cardiovascular diseases and arsenic intake in the UK Bangladeshi population.

6. Acknowledgments

This project has been funded, as part of the AquaTRAIN Marie Curie Research Training Network, by European Commission Sixth Framework Programme (Contract No: MRTN-CT-2006-035420). We would also like to thank the volunteers who took part in this study providing urine samples and completing questionnaires.

Chapter 4

URINARY TRACE ELEMENTS AS A FUNCTION OF ETHNICITY AND OTHER FACTORS IN THE UK

1. Introduction

Bio-monitoring studies on general populations are valuable tools for assessing the state of health and exposure to environmental contaminants, providing background knowledge for policy making and development of regulations.

Specific bio-monitoring studies on urine for the estimation of toxic/essential trace element content aim to set baseline levels in the general population living in a geographic area and to evaluate exposure to geogenic (Chowdhury *et al*., 2003; Lindberg *et al.,* 2008) or anthropogenic processes such as mines or industry (Banza *et al.,* 2009).

National and international sampling campaigns are undertaken by governments and international bodies as part of a regular monitoring process or as one-off projects. For instance, in the US, trace element baseline levels for the general population are regularly monitored within the National Health and Nutrition Examination Survey (NHANES)[12]. This is a program of studies designed to assess the health and nutritional status of adults and

[12] (http://www.cdc.gov/nchs/nhanes.htm).

children. In Europe some studies are carried out at a national level, including for Italy (Alimonti *et al.,* 2010) and Germany (Heitland and Köster, 2006), but a regular coordinated monitoring activity is still lacking in the EU compared to the US. The last European bio-monitoring project to determine and compare trace elements in biofluids in different EU regions started in 1992, was named the "Trace Element Reference Values in Human Tissues" (EUROTERVIHT) project (Sabbioni *et al.*, 1992), and highlighted significant differences related to geography and diet among different European countries. Finally, more recently a Consortium to Perform Human Biomonitoring on a European Scale (COPHES) was formed in 27 European countries with the aim of determining reference values of exposure in order to support policy making by evaluation for potentially hazardous environmental stressors. A first pilot study focused on mercury and cadmium in children and their mothers (Becker *et al.*, 2011).

The UK was involved in EUROTERVIHT, with a bio-monitoring study carried out for 16 trace elements and the analysis of about 200 samples of urine and blood from healthy volunteers (White and Sabbioni, 1998). Established reference values for urine in the UK are shown in Table 4-1. Another bio-monitoring project carried out to set reference values for whole blood, plasma and urine on levels of trace elements in the general population was previously carried out by the Supraregional Trace Element laboratories of the UK (Walker, 1992). These valuable studies (Walker, 1992; White and Sabbioni, 1998) are probably no longer representative of the current situation since more than 10 years have passed and environmental conditions and demographics have changed. A more recent pilot study was conducted on 400 volunteers in the UK and reported urinary cadmium and mercury levels only (Levy *et al.,* 2007).

Table 4-1: Urine reference values for some trace elements set in the EUROTERVIHT for the UK and for Italy; values are given in µg/L.

Country	Parameter	As	Se	Zn	Cu	REFERENCE
UK	MEDIAN	3.65	16.2		11.7	(White and Sabbioni, 1998)
	REF VAL	0.7-19.4	6.0-43.3		4.7-29.3	(White and Sabbioni, 1998)
UK	REF VAL	<10	<30	300-600	<60	(Walker, 1992)
ITALY	REF VAL	2.3-31.1	2.1-30.9	266-846	4.20-50	(Minoia *et al.,* 1990)

It is important to note that very few bio-monitoring studies consider variations in trace elements in urine in relation to ethnicity. Different ethnic groups residing in the UK keep traditional habits and eat 'ethnic' food, often imported from their country of origin.

Additionally, specific practices, such as betel quid chewing (Al-Rmalli *et al.*, 2011a), eating sikor (Al-Rmalli *et al*., 2010) and/or eating greater quantity of rice (Chapter 3), alter the individual trace element daily intake and urinary excretion even in individuals sharing the same geographical area and exposed to the same environment.

Arsenic (As) is a non essential trace element with proven carcinogenic properties in its inorganic state for the bladder, the lungs and the skin (IARC, 2004). It might be responsible for liver cancer (Liaw *et al*., 2008; Liu and Waalkes, 2008). Arsenic induces keratosis and hyperpigmentation (Yu *et al*., 2007). Arsenic exposure may result in neuropathic effects in the adolescence (Tsai *et al.,* 2003), effects on memory and intellectual function (Calderon *et al.*, 2001), reproductive effects with increased foetal loss and premature delivery (Chakraborti *et al.*, 2003; Milton *et al.*, 2005), steatosis (Chen *et al*., 2004), cardiovascular diseases (Lee *et al*., 2002), ischemic heart diseases (Tseng *et al*., 2003b), carotid atherosclerosis, respiratory system effects such as chronic cough and chronic bronchitis (Milton and Rahman, 2002). Arsenic limits in drinking water in Europe and in the US are set at 10 ppb.

Selenium (Se) is an essential element for humans. It is part of the structure of selenium cysteine an amino acid present in several human enzymes including glutathione peroxidases. Selenium is part of Iodothyronine deiodinases, selenoproteins contributing to systemic or local thyroid hormone homeostasis (Koehrle *et al.*, 2005). A deficit in Se intake results in adverse effects on the human body, such as Keshan disease in China (Yang *et al.*1998), while overexposure to selenium results in the loss of hair and fingernails (Yang *et al*., 1983) and nervous system abnormalities. Among trace elements, selenium has the narrowest range between essentiality and toxicity; just one order of magnitude. WHO sets the upper limit of safe range for humans at 400 μg/day, while deficiency is thought to occur at levels lower than 40 μg/day (WHO, 1996).

Copper (Cu) is an essential trace element that is important for humans since it is part of a number of metal enzymes including cytochrome C oxidase and superoxide dismutase that are required for collagen elastin formation and catalyzing redox reactions (McClatchey, 1994). In the general population, food is the major source of copper intake. Among food stuff, Copper is found in particularly high concentrations in nuts (8 mg/kg), shellfish and offal (40 mg/kg) (EVM, 2003). Copper safe upper level established by the Expert Group on Vitamins and Minerals is 0.16 mg/kg bw/day (EVM, 2003). Copper is absorbed from the

small intestine by a mechanism shared with Zn. Cu is bound to proteins in the epithelium and then transported to the liver by albumin. Within 2 hours, 60-95% of copper is in the liver and in the hepatocytes where it is trapped and incorporated into proteins. Part of copper is incorporated into the structure of ceruloplasmin. Copper is distributed to the other tissues where it becomes part of the enzyme superoxide dismutase. The remaining copper is mainly excreted via the bile. Acute copper toxicity is rare and results from food or beverage contamination. Copper has emetic properties and unpleasant taste that prevent accidental or deliberate ingestion. Just a few data on chronic copper toxicity in humans are available. Indian childhood cirrhosis (ICC) is a fatal disorder associated with accumulation of massive levels of copper in the liver (Tanner *et al.*, 1979). According to one hypothesis ICC can be due to boiling and storing milk in copper containers. However, more recently the causative role of Cu has been dispelled (Sriramachari and Nayak, 2008) and stronger emphasis is placed on the role of genetics (Scheinberg and Sternlieb, 1994). Isolated cases of idiopathic copper toxicosis (ICT) have also been reported in non-Indian communities in the US and Europe (Weiss *et al.*, 1989). Ford *et al*. (2000) found that elevated serum copper concentrations to be associated with cardiovascular disease in US by analysing the Second National Health and Nutrition Examination Survey.

Zinc (Zn) is essential for biochemical, structural and functional reasons. It is estimated that 3000 of the hundreds of thousands of proteins in the human body contain zinc prosthetic groups (Human Metabolome Database)[13]. It is a cofactor of several human enzymes, it is a membrane stabilizer, and it is involved in anabolism and catabolism of carbohydrates, lipids, proteins and nucleic acids. Zn is also essential in the transcription and translation of DNA. The principal clinical features of Zn deficiency are growth retardation, impacts to metal health and delays in skeletal and sexual maturation (Digirolamo and Ramirez-Zea, 2009). Acute oral exposure to zinc compounds causes gastrointestinal distress (WHO, 1982) and the estimated fatal dose of zinc phosphide is 40 mg/kg body weight. An excessive zinc intake may affect the immune system; Chandra (Chandra, 1984) reported that 4.29 mg Zn/kg body weight/day for 6 weeks of zinc sulphate altered the mitogenic response of peripheral blood lymphocytes and the chemotactic and phagocytic responses of polymorphonuclear leukocytes. Chronic oral exposures to zinc at doses of > 2 mg Zn/kg body weight/day for 11-24 months, could result in anaemia, associated with

[13] http://www.hmdb.ca/metabolites/HMDB01303

hypoceruloplasminaemia and hypocupremia (Prasad *et al*., 1978; Porter *et al.,* 1977) (Hoffman *et al*., 1988). Overexposure to dietary zinc interferes with gastrointestinal absorption of copper, potentially leading to secondary copper deficiency. Biological signs of high zinc exposure result in a secondary copper deficiency; decreased erythrocyte superoxide dismutase activity; increased LDL cholesterol and decreased HDL cholesterol; decreased glucose clearance and abnormal cardiac function; abnormal cardiac function; and impairment of pancreatic enzymes, amylase and lipase (EVM, 2003).

There is evidence of health inequalities between ethnic minority communities living in the UK. According to a health survey for England carried out in 1999 (Primatesta and Brookes, 1999), for cardiovascular disease conditions (including angina, heart attack, stroke, irregular heart rhythm, reported high blood pressure and diabetes) all South Asian groups living in the UK showed higher rates than the general population for most conditions. Both Pakistanis and Bangladeshis showed higher rates than Indians. In particular, for diabetes Pakistanis and Bangladeshis of both sexes showed rates over five times higher than the general population, and Indians almost three times higher (Primatesta and Brookes, 1999).

In this chapter, urinary concentrations of As, Se, Cu and Zn are reported for 161 volunteers from the Bangladeshi, Pakistani and Indian communities residing in the UK and a White Caucasian group. Data are presented here and the results are rationalised in light of demographics, ethnicity and other factors including smoking, rice and fish consumption. This chapter provides an insight into factors modulating baseline levels of trace elements and contributes to setting background knowledge on how trace elements might impact on the state of the health of the general population in the UK.

2. Sample collection

Urine samples from adult volunteers living in the UK (England, mainly London and Leicester) were collected between 2008 and 2010. This study was approved by the Faculty of Health and Life Sciences Human Research Ethics Committee of De Montfort University and each volunteer gave an informed consent. Volunteers provided spot urine (mainly first morning) in a polypropylene bottle and filled in a questionnaire providing basic demographic information. Urine samples were frozen at -20°C after collection and kept frozen till the day of the analysis.

3. Materials

Materials and methods are the same as those described in Chapter 3.

3.1 Preparation and analysis of total trace elements in urine

Briefly, after defrosting and agitating, 2 ml of urine was digested with 1:1 (69.5% nitric acid and 30% H_2O_2 in a microwave, with an accelerated reaction system (MARS, Matthew Inc. USA) up to 95°C (power 600W, pressure at 120 PSI). After cooling, samples were diluted to 1:15 with MilliQ water. Analysis was performed with an ICP-MS 7500c (Agilent Technologies, USA). Polyatomic interferences are known to occur in urine, such as from the argide $^{40}Ar^{35}Cl^+$ on $^{75}As^+$ and $^{40}Ar^{37}Cl^+$ on $^{77}Se^+$. To minimise polynuclear interferences occurring in urine a collision cell with H^2 as collision gas (flow 2 mL/min) was used. Rh 10 µg /L was added *via* an inline channel. The monitored signals are shown in Table 4-2, seven replicate measurements were performed per sample. A set of standards including blanks were measured every 40 samples, along with the certified reference material (Trace elements in urine lot 0511454, Seronorm).

Table 4-2 : Analysed elements in urine and isotopes from this study

Element	Isotopes monitored
As	75
Se	77, 78
Cu	63, 65
Zn	64, 66
Rh	103

4. Specific gravity

Specific gravity of urine has been measured as described in Chapter 2.

5. Statistics

Raw data as single counts have been exported into excel and the concentrations were calculated. Statistical analysis has been performed in SPSS.

6. Results

6.1 Quality controls

Counts were exported from the ICP-MS instrument and processed in Microsoft Excel in order to determine trace element concentrations. An example of a calibration curve and table with counts and concentrations in the chapter's Appendix, (Figure A4-1 and Table A4-1). An external calibration was used. Calibration curves were linear with R^2: 0.99 - 1. In order to correct for signal drift, rhodium was used as an internal standard. However it was verified that Rh correction for ^{75}As and ^{78}Se yielded a pronounced over-recovery on the CRM: ^{75}As (119%, RSD: 6.2%) and ^{78}Se (135% and RSD: 2.4%). Therefore for these two elements, no Rh correction was adopted, allowing a recovery of 90-99 % and 106-107% for As and Se respectively. In contrast, results for Zn (66 and 64) and Cu (63) were satisfactory when corrected using Rh counts and therefore, this approach was adopted for these elements. Samples were analysed in two separate runs (one and two). Recoveries for CRM analysis are shown in Table 4-3, for runs one and two.

The Limit of Detection (LOD) was calculated as three times the standard deviation of the procedural blanks and multiplied by the dilution factor according to the European standard prEN 13804. LODs for As, Se, Cu and Zn were 1.66, 2.12, 10.6 and 13.7 µg/L respectively. Samples below LODs have been substituted with LOD/2 to allow statistical analysis.

Table 4-3: Recovery from CRM for run one and run two, certified reference materials are shown in the measurement order

Run I					
Sample ID	CRM%	**^{75}As [µg/L]**	**^{78}Se [µg/L]**	**^{63}Cu [µg/l]**	**^{66}Zn [µg/L]**
crm77		161.1	65.6	79	1225
crm136		142.5	52.6	114	1239
crm202		139.2	68.5	66	1021
crm256		140.4	62.9	88	1173
crm307		140.4	60.7	61	1025
	average value	**140.6**	**62.1**	**82**	**1137**
	expected value	**142**	**58.6**	**78**	**1141**
	% recovery	**99**	**106**	**105**	**100**
Run II					
Sample ID	CRM%	**^{75}As [µg/L]**	**^{78}Se [µg/L]**	**^{63}Cu [µg/l]**	**^{66}Zn [µg/L]**
crm20		147.3	75.4	92	1395
crm369		104.8	50.1	76	982
crm448		129.3	57.7	81	1145
crm527		136.1	65.9	77	1084
crm528		140.8	65.8	80	1089
	average value	**127.8**	**63.0**	**81**	**1139**
	expected value	**142**	**58.6**	**78**	**1141**
	% recovery	**90**	**107**	**104**	**100**

For copper, both 63 and 65 isotopes were monitored. A greater contribution of ^{65}Cu was noticed in run one (147%) than in run two (113%). This was possibly the result of an incomplete matrix removal. However, since isotope 63 was not affected, the use of this isotope was preferred for calculations as also reported elsewhere (Heitland and Köster, 2006). For zinc, no difference between isotope 66 and 64 was noticed and therefore isotope 64 was used for the calculations.

6.2 The effect of specific gravity correction

Urine is affected by variation in dilution in relation to water intake and glomerular filtration. In order to account for individual urine variation, specific gravity was used to normalise and to allow inter-individual comparison. The formula used to correct is the one used by (Nermell *et al.*, 2008a):

U As SG [µg/L] = (Urinary Arsenic [µg/L] x averageSG-1)/(Specific Gravity - 1.000) (Equation 6)

The average SG used was 0.016. This formula has been used for all the 4 elements presented in this study. The SG correction effect on As concentration is shown in Figure 4-

1. The correction tends to affect outliers most strongly, with relatively little affect on values around the average (1.016 in this case). Urinary arsenic is usually not normally distributed (as seen in Chapter 3 and in Chapter 6) and this trend is left after SG correction, requiring the use of non-parametric statistics.

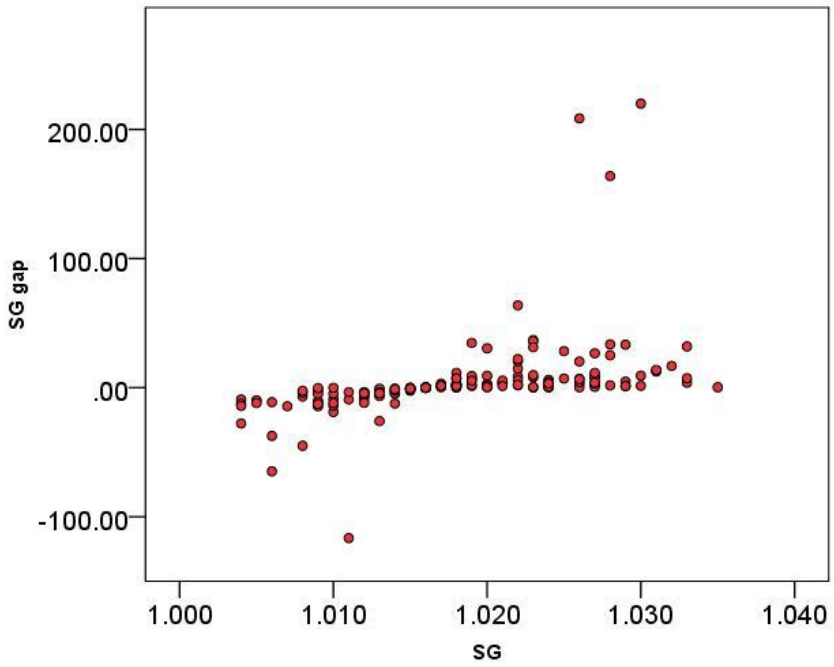


Figure 4-1: The effect of SG correction on the difference between unadjusted and adjusted urinary arsenic (gap) for 161 urine samples. SG correction has a greater effect on samples on the higher and lower range of distribution.

6.3 The effect of ethnicity

A total of 161 unique spot urine samples of volunteers residing in the United Kingdom were analysed in this study and the descriptive statistics are shown in Table 4-4. Trace elements in urine were not normally distributed, therefore median and 25th and 75th percentile are presented.

In Table 4-5 data are presented in relation to ethnicity for Bangladeshi (B) *n*=54, Indian (I) *n*=25, Pakistani (P) *n*= 21 and white (W) *n*=23 groups residing in the UK. A group of 38 volunteers (indicated as 'O') did not belong to these latter ethnicities or did not specify their ethnic background on the questionnaire.

Table 4-4: Trace element levels in the overall study group from the UK*

***n*=161**	**^{75}As**	**^{78}Se**	**^{63}Cu**	**^{64}Zn**
median	15.2	15.5	17.5	362
25th percentile	6.3	9.7	9.8	256
75th percentile	32.8	21.0	28.2	525

*Values are expressed in μg/L and corrected by specific gravity

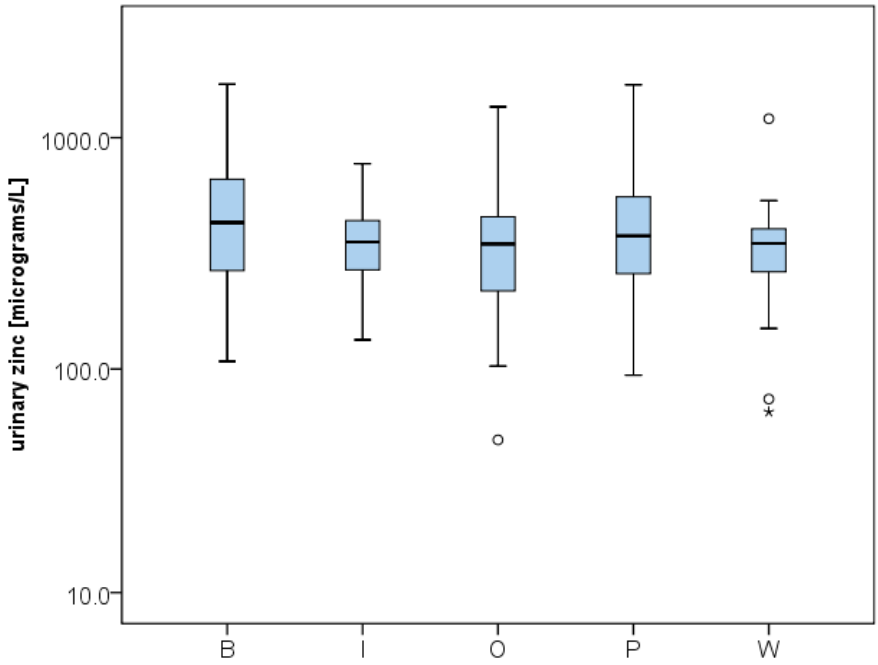
Table 4-5: Trace element levels in urine of different ethnic groups in the UK

BANGLADESHI (*n*=54)	**^{75}As**	**^{78}Se**	**^{63}Cu**	**^{64}Zn**
median	23.6	17.6	30.2	430
25th percentile	15.9	14.0	25.4	268
75th percentile	37.2	22.4	39.5	654
PAKISTANI (*n*=21)	**^{75}As**	**^{78}Se**	**^{63}Cu**	**^{64}Zn**
median	5.6	4.1	15.6	377
25th percentile	0.9	2.7	7.7	259
75th percentile	16.4	13.6	24.6	556
WHITE (*n*=23)	**^{75}As**	**^{78}Se**	**^{63}Cu**	**^{64}Zn**
median	11.2	18.9	10.5	350
25th percentile	6.7	7.2	10.0	267
75th percentile	23	24	14	405
INDIAN (*n*=25)	**^{75}As**	**^{78}Se**	**^{63}Cu**	**^{64}Zn**
median	5.8	13.8	14.8	355
25th percentile	3.6	9.0	9.3	269
75th percentile	12	16	20	439

*Values are expressed in μg/L and corrected by specific gravity, 38 volunteers of other ethnic origin are not reported in this table

Figures 4-2 and 4-3 show box plots for the analysed trace elements in urine according to ethnicity. Since the variables were not normally distributed, a Kruskal-Wallis test was used to screen for differences among the ethnic groups. Significant differences between

A)



B)

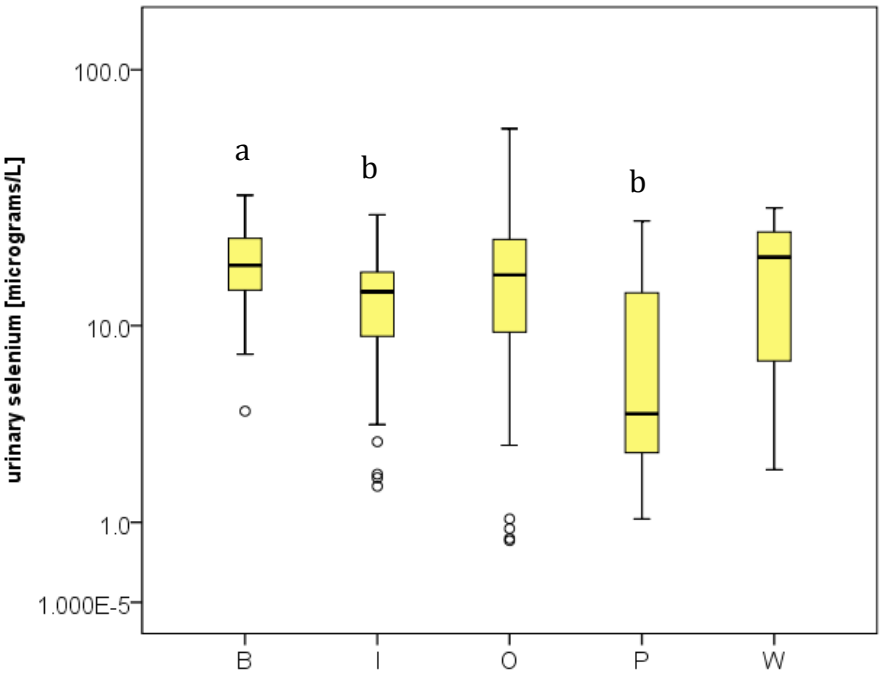
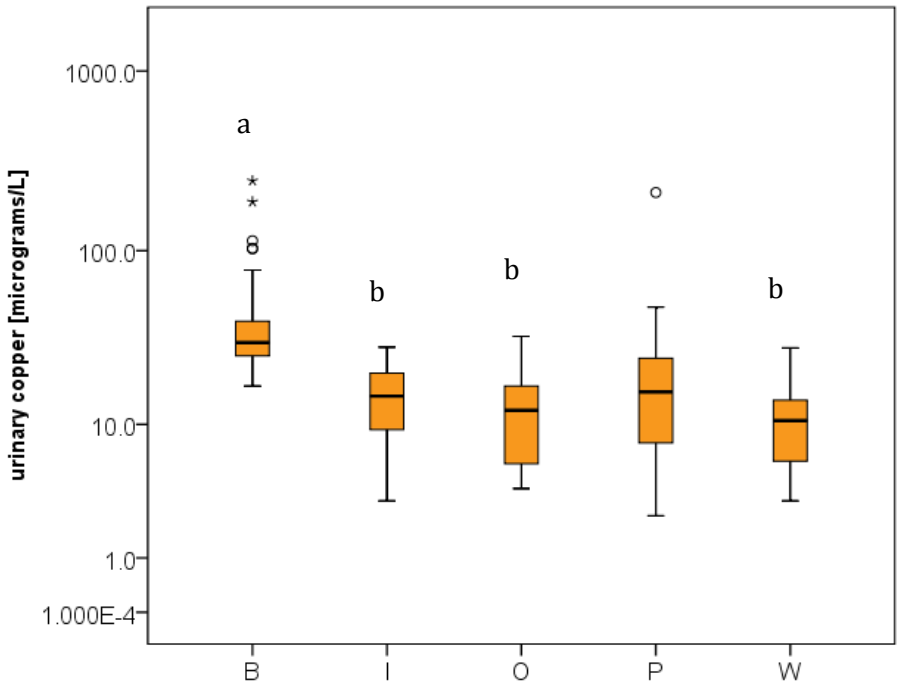


Figure 4-2: Box plots of urinary zinc (plot A, p= 0.223) and selenium (plot B, p<0.001) levels in the urine of the ethnic groups: Bangladeshi (B), Indian (I), Pakistani (P) and White Caucasians (W) and others (O).

A)



B)

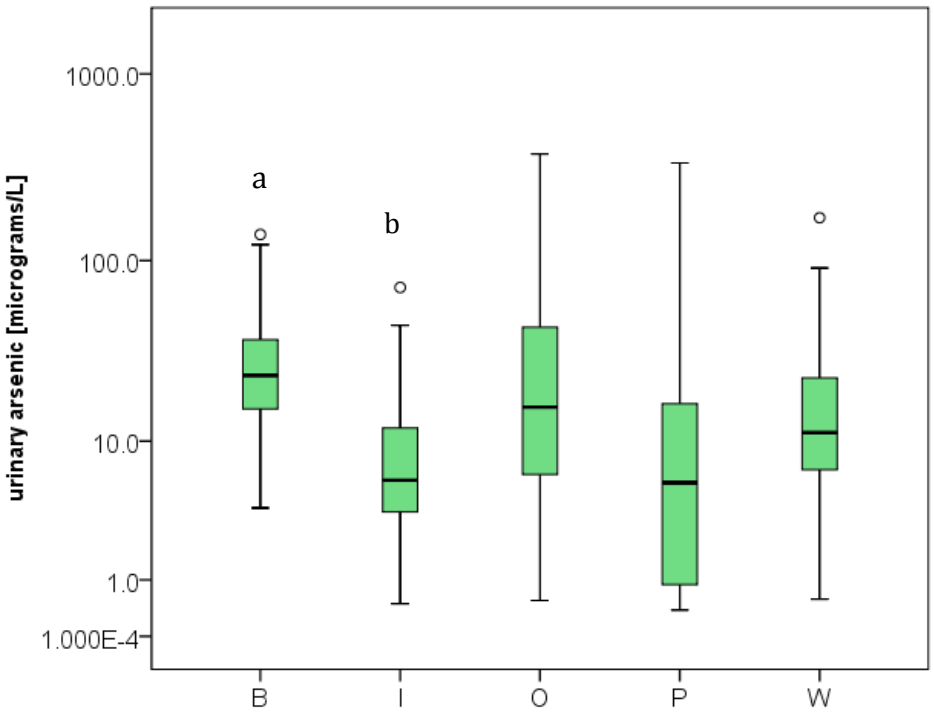


Figure 4-3: urinary copper (A, p< 0.001) and arsenic (B, p< 0.001) in urine of different study groups Bangladeshi (B), Indian (I), Pakistani (P) and White Caucasians (W) and others (O). Circles represent cases with values between 1.5 and 3 box lengths from the upper or lower edge of the box. Stars represent cases with values more than 3 box lengths from the upper or lower edge of the box. The box length is the interquartile range.

the ethnic groups were found for As ($p<0.001$), Se ($p<0.001$) and Cu ($p<0.001$). Furthermore, a Dunnet post-hoc test was run to identify intergroup differences. With this approach, urinary As was found to be significantly higher in Bangladeshis than Indians, Se levels for Bangladeshis were significantly higher than Indians and Pakistanis. Also evident from the graph is the significantly higher urinary Cu for UK Bangladeshis compared to Indians, white or the "other" group.

The ratio of urinary copper to zinc was calculated for the 161 volunteers (see Figure 4-4) and compared with the Kruskal-Wallis test, which confirmed the presence of a significant difference ($p< 0.001$) with an evident increase for Bangladeshis compared to other groups.

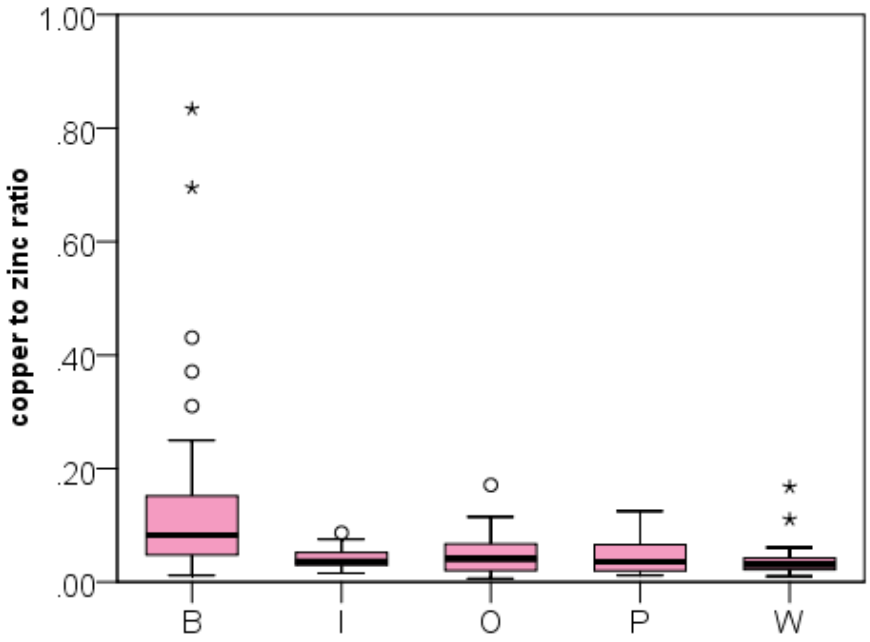


Figure 4-4: Urinary copper to zinc ratio calculated for the 161 volunteers and grouped according to ethnicity.

6.4 A focus on the UK Bangladeshi group

The Bangladeshi group was the most populous in this study and the one that has shown the most distinct urinary trace element composition, especially for Cu. In order to investigate the reason for the higher urinary copper levels in the UK Bangladeshi group, a number of factors were considered.

Betel quid chewing (also commonly called as chewing pan) is common in the Bangladeshi community (Al-Rmalli *et al.* 2011). For the population studied as part of this project, it was found to be prevalent in 21 out of the 54 volunteers, 17 reported not to chew and 16 did not provide information. No statistically significant difference was found when comparing chewers with non chewers for any of the elements on a Mann-Whitney test.

Twelve volunteers out of 54 reported as being diabetic, 25 reported not to be diabetic and 17 did not provide information on this question. On a Mann-Whitney test, when screening for differences in urinary trace element content of diabetic versus the non-diabetic group, a significant difference for zinc (P= 0.005) was detected. The median of urinary zinc for the diabetics was 717 µg/L *versus* 412 µg/L for the non-diabetic volunteers. A box plot is shown in Figure 4-5. No significant difference in urinary copper was found between diabetics and non-diabetics.

The use of medications in the Bangladeshi group did not significantly affect any of the analysed trace elements or the copper to zinc ratio in this study group, as demonstrated by the Mann-Whitney test. Upon multivariate analysis (PLD-SA), the Bangladeshi group showed some separation, with a predictable big influence from urinary copper as shown in Figure 4-6 A and B.

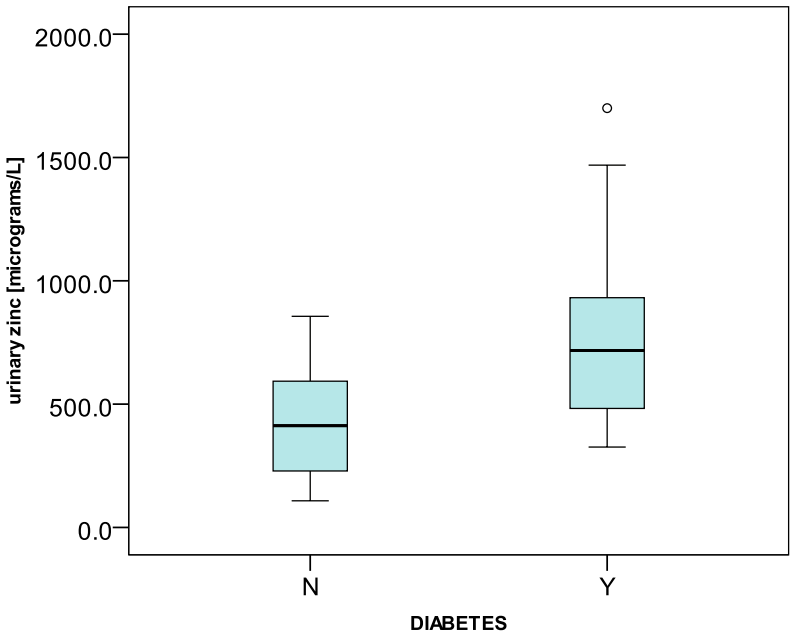
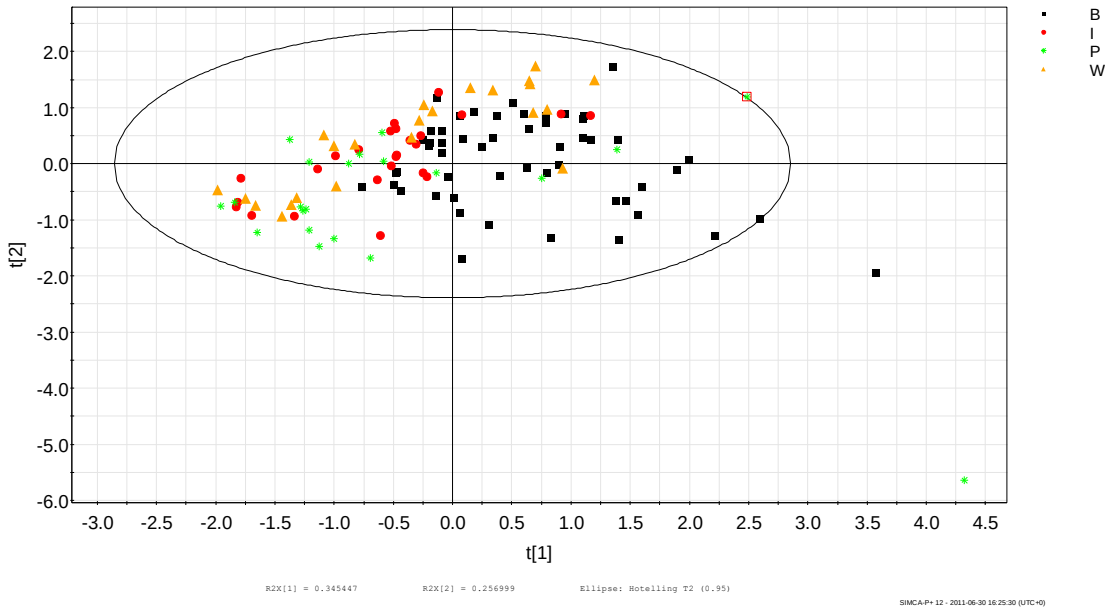


Figure 4-5: Urinary zinc is higher for UK Bangladeshis affected by diabetes (Y) than in not affected (N) (P=0.005).

A)



B)

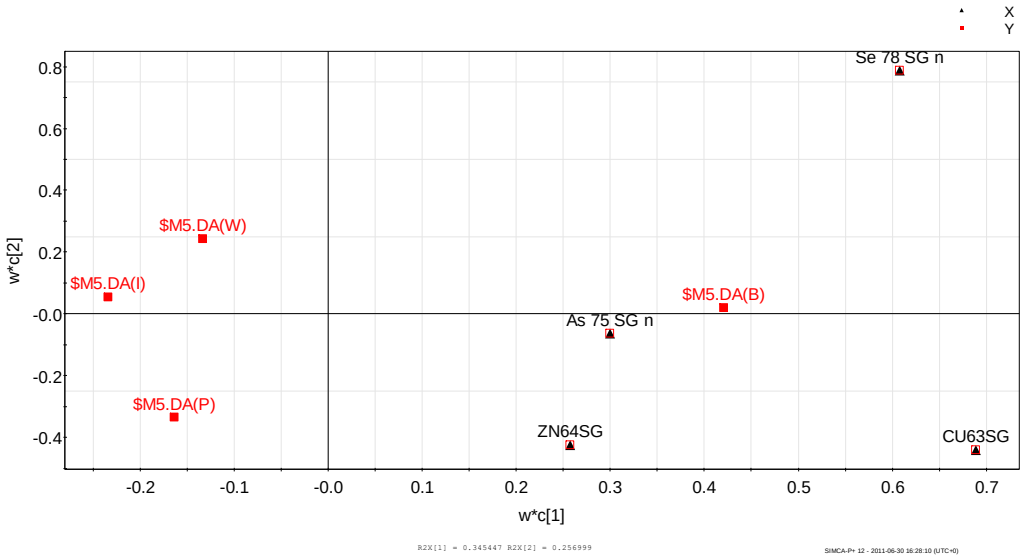


Figure 4-6: Score scatter plot (A) and loading scatter plot (B) obtained in SIMCAP for urinary trace elemental profile of Bangladeshis (B), Indians (I), Pakistani (I) and white Caucasians (W). Note the contribution played by copper (CU63SG) in driving the division of the B group from the rest of the study population.

6.5 Age and gender

A total of 150 volunteers (93%) provided information on gender in the questionnaire. Within the dataset, 45 volunteers declared to be females and 95 to be males; for the remaining ten volunteers gender was not specified.

No significant difference related to gender for urinary arsenic (P=0.816), zinc (0.262) copper (0.594), and selenium (0.70) was detected on a Kruskal-Wallis test.

Information on age was provided by 120 (75%) volunteers. In order to address any effect of age on urinary trace element composition, a Spearman test was run. Correlation coefficients and significance are reported in Table 4-6. A highly significant positive correlation (p< 0.001, r: 0.443) was detected for copper (see scatter plot in Figure 4-7). A significant positive correlation with age was also seen for As and Se as shown in Table 4-6.

Table 4-6: Study of correlation (Spearman test) between age and urinary elements

	r	P
Cu and Age	0.443	<0.001
As and Age	0.306	0.001
Se and Age	0.219	0.016
Zn and Age	0.085	0.353

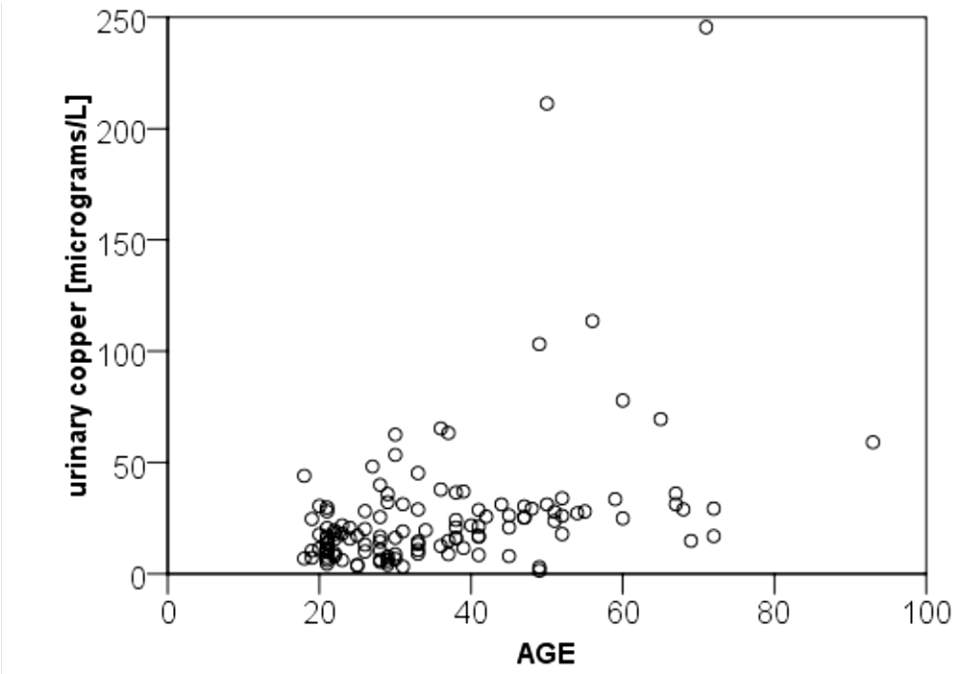


Figure 4-7: Scatter plot of urinary copper in the overall population and age

6.6 Smoking

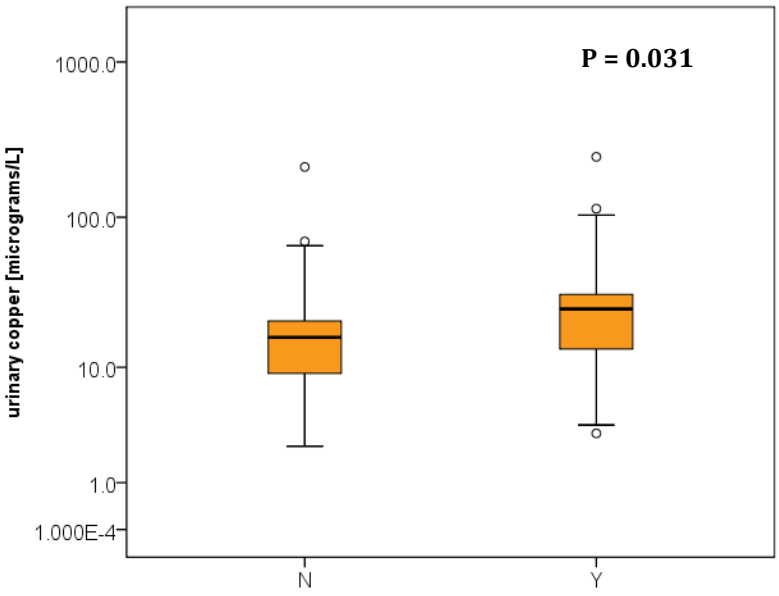
Just 16 out of 161 people in this study group declared themselves as smokers; 44 did not provide information and the remaining 101 were non-smokers. By means of a non parametric test, the differences in urinary trace element composition as a result of smoking was tested. For arsenic, selenium and zinc no significant difference was found. For copper, a slight but significant (p = 0.013) decrease in urinary copper was seen in smokers (median: 16.4 µg/L in smoker's *vs*. 20.2 µg/L in non smokers).

6.7 Fish consumption effect on arsenic and copper

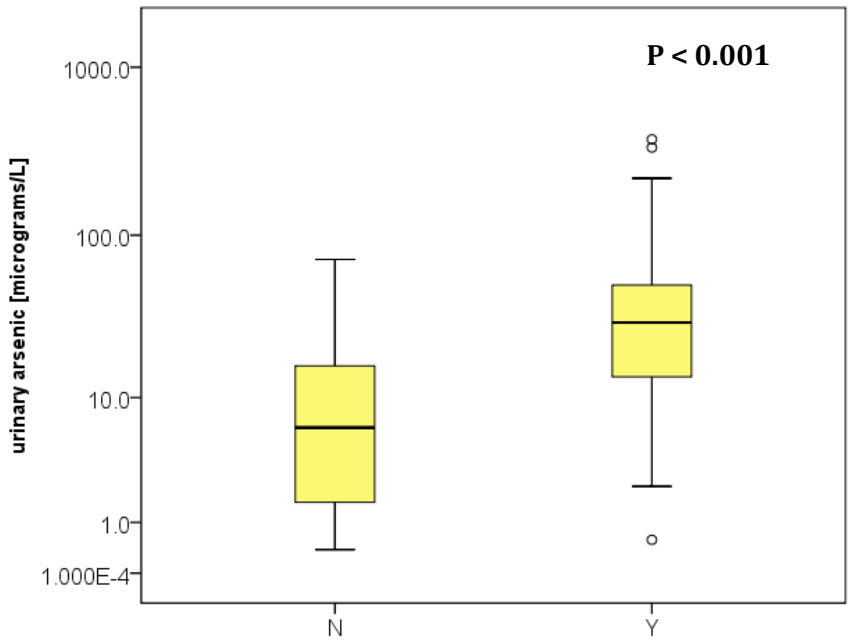
In this study, volunteers were not asked to refrain from eating fish or other types of food before urine collection. However, they were invited to provide a food diary and other information for the two days before urine collection.

Among the volunteers providing information on fish (101 out of 161), 64 had consumed some type of fish in the two days before urine collection (both sea and fresh water fish) and 37 declared that they did not eat fish during that period. Figure 4-8 A shows the urinary copper and arsenic in the study group that had fish within the two days of urine collection (Y) and those who declared not to have eaten fish within the 2 days of urine collection (N).

A)



B)



C)

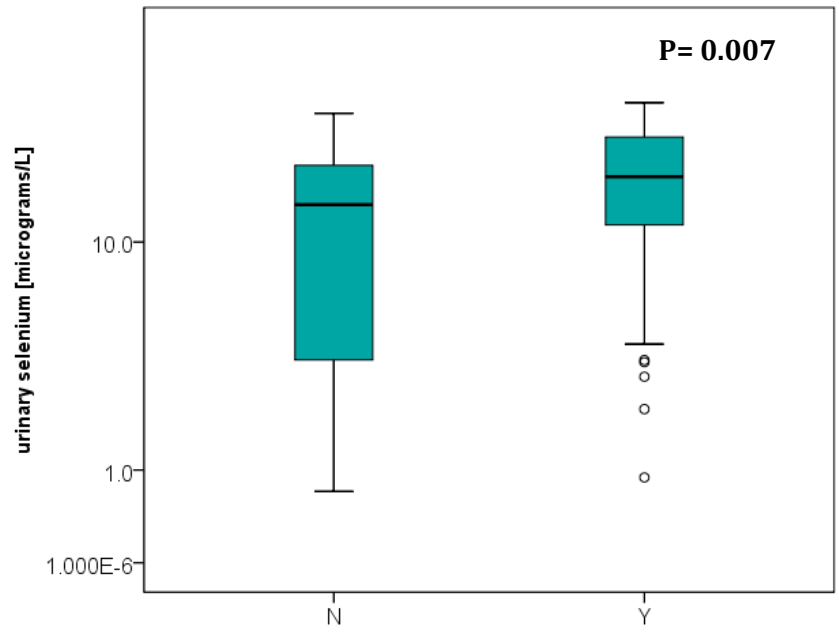


Figure 4-8 A) Urinary copper (A), arsenic (B) and selenium (C) in people who had fish (Y) and those who declared not to have eaten fish (N) in the two days before urine sampling. SG corrected values.

A non parametric test (Kruskal-Wallis) on these three groups yielded a highly significant variation for arsenic ($p < 0.001$), selenium ($p= 0.007$) and for copper ($p =0.031$). In all the three cases, fish eaters (Y) had higher urinary levels of each of these elements than those who declared not to have eaten fish (N). The copper median for the N group was 16.1 µg/L and 25.0 µg/L for the Y group. The same trend was seen for arsenic with 6.3 µg/L and 29.7 µg/L median concentrations for N and Y respectively. The difference for selenium (13.5 µg/L N *vs* 16.9 µg/L Y) was less pronounced.

6.8 The effect of rice consumption on urine urinary trace elements

For a subgroup of 40 volunteers belonging to the Bangladeshi and White group, data on daily rice consumption were available. In these cases, correlation between grams of rice eaten per day and total urinary trace elements were tested by means of a Spearman test. The only significant correlation was the one between total copper and rice. Table 4-7 reports P and Rho values and Figure 4-9 presents a scatter plot for urinary copper and daily rice intake.

Table 4-7: Correlation between rice consumption and urinary total trace elements (*n*=40)

Rice Consumption [g/day]	Se	As	Cu	Zn
Rho	-2.11	0.179	***0.552***	0.207
P	0.190	0.269	***0.001***	0.200

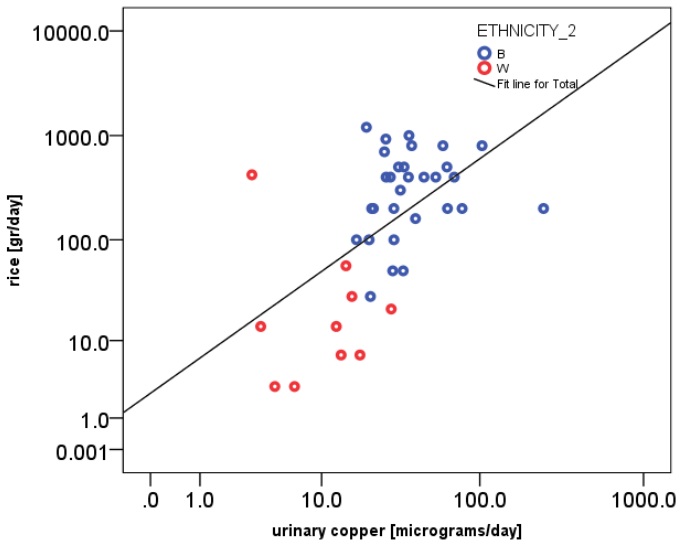


Figure 4-9: Scatter plot of urinary copper *vs* rice intake

6.9 Selenium correlates to arsenic in urine

In order to monitor the correlation of the 4 trace elements analysed in urine a Spearman test was run using the dataset of the 161 volunteers. The test revealed a highly significant positive correlation between urinary arsenic and selenium, arsenic and copper, selenium and zinc and copper and zinc, as shown in Table 4-8 and Figure 4-10. The strongest correlation was between total arsenic and selenium with a r=0.608 and a p< 0.001.

In Figure 4-11 the arsenic versus selenium trend is shown in relation to fish consumption. There was a different pattern for volunteers who had no fish in the two days before urine collection and a decrease in the slope for those who had eaten fish.

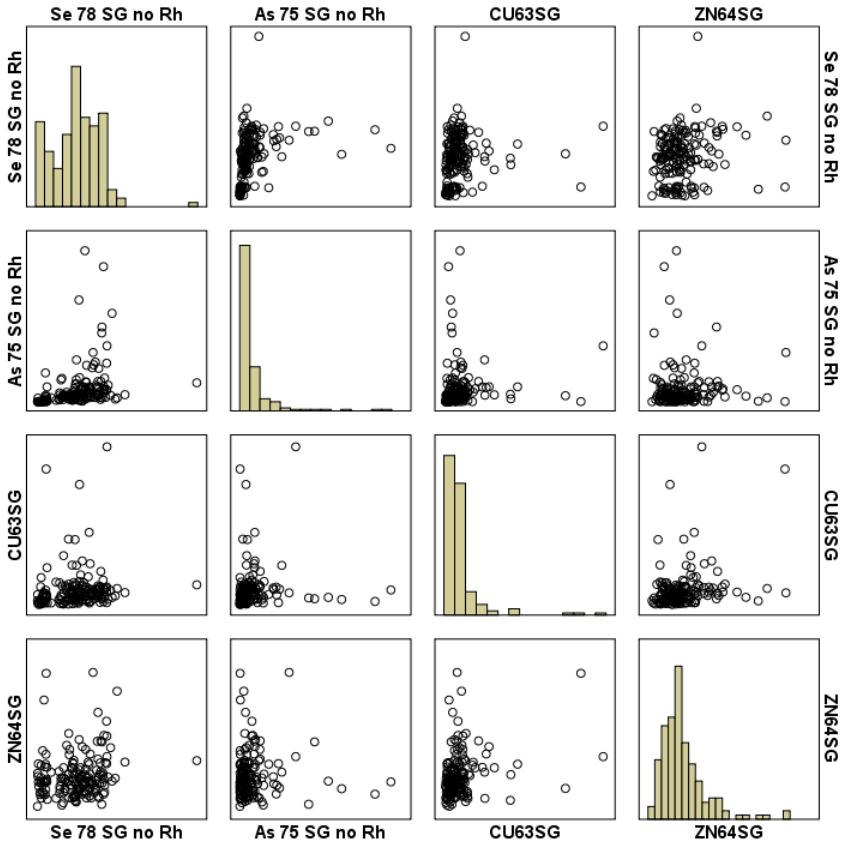


Figure 4-10: Matrix plot for urinary trace elements in the study group (n=161 volunteers) form the UK. Used isotopes are indicated in brackets. All values are SG corrected.

Table 4-8: Correlation between trace elements in urine of 161 volunteers residing in the UK.

Correlation	Spearman's rho	Significance
As vs Se	0.608	<0.001
As vs Cu	0.283	<0.001
Zn vs Se	0.197	0.012
Cu vs As	0.276	<0.001
As vs Cu	0.313	<0.001

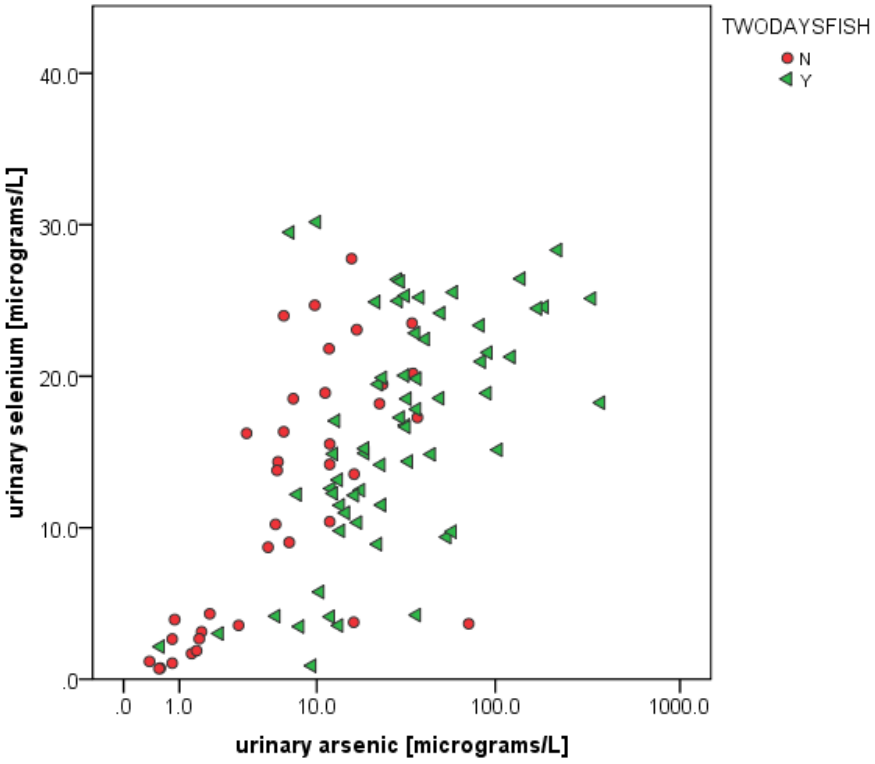


Figure 4-11: Positive correlation of urinary arsenic and selenium in the UK volunteers. Volunteers who had fish in the two days before urine sampling (TWODAYSFISH=Y) are represented by red circles and volunteers who had no fish (TWODAYSFISH=N) by green triangles. Urinary arsenic and selenium have been corrected by specific gravity; arsenic is on a log scale.

7. Discussion

In this study, urinary trace element composition for arsenic, selenium, copper and zinc for a group of 161 volunteers from the UK is presented. Urinary levels are considered for the whole UK population and split into four different ethnic groups: Bangladeshi, Indian,

Pakistani and White Caucasians. Additionally, the effect of factors of gender, age, smoking and fish consumption are considered.

Ethnicity was found to impact on urinary copper of volunteers residing in the UK; the Bangladeshi group had about 2.8 times higher urinary copper than White Caucasians (medians: 30.2 μg Cu/L versus 10.5 μg Cu/L). Pakistani, Indian and 'Other' groups were characterised by intermediate urinary copper values between the Bangladeshi and the White Caucasians. It is interesting to note that the median for the white Causasian group (10.5 μg Cu/L) was very similar to that previously reported for the UK population of 11.7 μg Cu/L (White and Sabbioni, 1998), while the median of the UK Bangladeshi group exceeded the upper limit of the reference value set at 29.3 μg/L for the UK population. Some very high urinary copper values were found in the UK Bangladeshi group and for one of the Pakistani volunteers. Calculations performed after excluding the outliers still yielded a significant difference in urinary copper in the studied ethnic groups.

Possible reasons for the increased urinary copper in the Bangladeshi study group compared to the white Caucasians might be (1) higher consumption of foods rich in copper (e.g. offal, shellfish and rice); (2) the use copper-made utensils for cooking and boiling water; (3) the presence of a different copper species that is not able to be eliminated through the bile but is excreted through urine; (4) an additive source of copper intake such us chewing betel quids (pan); or (5) related to some genetic or disease state. From the data presented, it is evident that chewing pan did not significantly affect any urinary trace element levels in the Bangladeshi group, including copper. Neither smoking nor the intake of medications caused an increase in urinary copper levels. However, among the factors here considered, rice did have an impact. Rice daily intake was highly significantly ($p < 0.001$) positively (rho=0.522) correlated to urinary copper in 40 volunteers from the Bangladeshi and White groups. Offal was not frequently nominated in the food diary of the Bangladeshi group. Fish consumption, was very spread in the Bangladeshi group, and accompanied by an increase of about 1.5-fold urinary copper in those volunteers that had consumed fish in the two days before sampling. The consumption of fresh water fish (imported from Bangladesh) was very common among this group as also discussed in Chapter 3. Other additive factors possibly playing a role in such urinary copper in UK-Bangladeshis compared to white Caucasians might be related

to the state of health of the Bangladeshi group (see below). Diabetic Bangladeshis did not show an higher copper in urine.

According to the information sampled in this study, both rice and fish consumption seemed to contribute to the observed increase of urinary copper levels in the Bangladeshi study group compared with other volunteers from this study and with references cited for the UK population (White and Sabbioni, 1998). However, the possible role of genetics cannot be ruled out as well as the presence of other factors not screened in this study.

South Asian groups living in the UK showed higher rates of cardiovascular disease in comparison with the general population. Both Pakistani and Bangladeshi UK groups showed higher rates than UK-based Indians (Primatesta and Brookes, 1999). Elevated serum copper concentrations were associated with cardiovascular disease in the US from data from the Second National Health and Nutrition Examination Survey (Ford, 2000). According to Ford, high copper concentrations may be related to coronary heart disease in at least two ways: i) by oxidizing low density lipoprotein cholesterol and increasing its atherogenicity and ii) by promoting inflammation that accompanies coronary heart disease. Therefore, the increase in urinary copper in the Bangladeshi group might be related to the increase in incidence of cardiovascular disease and requires future epidemiological investigations.

In contrast to copper ($p < 0.001$), zinc was not affected by ethnicity ($p=0.223$). After the exclusion of diabetic volunteers, Zn was even less different among ethnicities ($p = 0.712$). However, the diabetic UK Bangladeshi group had significantly higher levels of zinc in urine compared to the non-diabetics. The increase in urinary zinc of diabetic patients is consistent with findings from several studies on animal models and humans (Canfield *et al.,* 1984, Cunningham *et al.,* 1994, Dettwyler, 1966, Kiilerich *et al*., 1990, Kinlaw *et al.,* 1983, Lau and Failla, 1984, Levine *et al.,* 1983, McNair *et al.,* 1981, Pai and Prasad, 1988, Kazi *et al.,* 2008). There is a complex relationship between diabetes, insulin and Zn with unclear cause and effect relationships; zinc is part of the chemical structure of insulin, but until now, the cause of the hyperzincuria in diabetic animals and humans has not been clearly identified. Furthermore, insulin zinc suspension mixed bovine insuline is a medication which is used in diabetes mellitus and might increase the urinary levels of

Zn[14]. It is not known whether the diabetic volunteers from this study had this type of medication administered. After a review of more than 230 research studies, Jansen (Jansen *et al.,* 2009) concluded there is an involvement of zinc in multiple processes of the development and progression of diabetes mellitus, emphasizing the diabetogenic effect of a disturbed zinc homeostasis. Unfortunately, no urinary zinc values were reported by (White and Sabbioni, 1998)for the UK population. However, a comparison to the Italian reference values by (Minoia *et al.*, 1990) of 266-846 µg Zn/L, and to previous published data for the UK as shown in Table 4-1, shows that zinc in the study population is compatible with existing literature, with diabetic people at the end of distribution and one outlier in each group (not visible in the log scale graph in Figure 4-2 A).

Urinary arsenic showed very broad variation as illustrated in Figure 4-3 B. As discussed in Chapter 3, it is well known that total urinary arsenic accounts for dietary exposure to organic species of arsenic, such as arsenobetaine (AB), that are not directly linked to toxic effects in humans and are the consequence of recent consumption of seafoods or poultry. Therefore, total arsenic in urine is not sufficient *per se* to draw conclusions on arsenic risk assessment. Volunteers did not refrain from eating fish before urine collection. Total arsenic values reported for the UK group were significantly affected ($P < 0.001$) by fish consumption. Volunteers who declared to have consumed fish within the two days prior to urine sampling showed significantly higher total urinary arsenic level in this study; the median for the group having eaten fish (Y) was 29.7 µg As/L *versus* 6.3 µg As/L for the one that declared no fish consumption (N). It is assumed that the N group is representative to set a total arsenic baseline in the UK; the arsenic level of the N group was within the range 0.7-19.4 µg As/L set by White and Sabbioni (1998), but the median was higher than 3.65 µg As/L which was reported by the author. Feldmann's group (Newcombe *et al.*, 2010) has shown that even after 5 days of controlled diet with no fish intake, a certain amount of arsenobetaine is still released from the body (from 0.2 to 12.2 µg As/L), suggesting either that AB accumulates in the tissues, or that AB is a human metabolite of dimethylarsinic acid or inorganic arsenic from the trial food given to volunteers. Therefore, arsenobetaine or other arsenic species could still contribute to the total urinary arsenic in the UK study group even from those volunteers that declared no fish consumption.

[14] http://www.medicines.org.uk/guides/insulin%20zinc%20suspension%20mixed%20bovine/diabetes

Selenium levels reported here for the overall study population are within the range for the UK population (White and Sabbioni, 1998) for all the considered ethnicities. Selenium was higher in volunteers that had fish within two days before urine collection and was strongly positively correlated to total arsenic in urine. A different pattern and slope is evident for the Y and N groups. This might imply that arsenic and selenium are excreted together in urine as conjugated chemical forms, such as glutathione compounds (Gailer *et al.*, 2000b). However, after fish consumption the excretion of 'arsenic only species' (such us AB and DMA) dominates, changing the slope of the curve. This trend has been also observed in a review of literature form the world by Brima (private communication).

The copper to zinc ratio was significantly different in the ethnic groups studied, with a significant increase in Bangladeshis compared to Caucasians. Within the Bangladeshi population however, copper to zinc ratio was lower in the UK Bangladeshis affected by diabetes in comparison to non-diabetics as a consequence of the higher urinary zinc content in the former group. In the elderly, serum copper to zinc ratio is associated with the physical decline and baseline physical and functional measures, and subjects in the high copper to zinc ratio tertile had a higher risk of death (Mocchegiani *et al.*, 2011).

8. Limitation of this study

The main limitation of this study lies in the small number of volunteers and therefore the initial findings will need validation through a large scale research project by means of an epidemiological study with better stratification of the population into ethnic groups.

9. Conclusions

Data presented in this chapter demonstrated different urinary copper levels in the different ethnic groups living in the UK. The most peculiar urinary profile was found for the UK Bangladeshi group with a marked increase in urinary copper in comparison to white Caucasians and other groups from this study, as well as compared to previously determined levels for the UK population. Amongst the reasons for this increase is the possible role of diet and particularly rice and fish consumption amongst the Bangladeshis. It is important to clarify whether or not this increase in copper in urine, along with the higher copper to zinc ratio in the Bangladeshi study group, has an impact on the reported increase of cardiovascular disease in the UK-Bangladeshi population, and general health

inequalities for this community. Zinc was found to be higher in urine from diabetic volunteers in this study and this is in line with what has been reported in the literature.

Selenium strongly correlated to arsenic in urine with different behaviours before and after fish consumption. As and Se might be excreted in a common chemical form.

10. Acknowledgments

I would like to thank all the volunteers who donated the urine samples for this study and Adilah Mulla and Raees Vanker for facilitating sampling process for the Pakistani and Indian group.

Chapter 5

URINARY METABOLOMICS AS A FUNCTION OF DIET, ARSENIC AND SELENIUM EXPOSURE

1. Introduction

Arsenic is a class I carcinogenic agent that accounts for about 20% of mortality in Bangladesh (Argos *et al.*, 2010). Human bio-monitoring studies to assess the health impact of chronic exposure to arsenic mainly rely on the detection of total arsenic and arsenic species as biomarkers of exposure in urine (Morton and Mason, 2006; Cascio *et al.,* 2011), blood (Li *et al.*, 2011), hair and nails (Brima *et al.*, 2006b). Some groups have applied genomics (Ghosh *et al.*, 2008) and more rarely proteomics (Navas-Acien and Guallar, 2008) to identify markers of arsenic sensitivity and effect. A few studies have applied metabolomics to demonstrate the effects of arsenic exposure on animals such as bank voles (Griffin *et al*., 2001). One study on rats (Wei *et al.,* 2009) documented urinary, serum and liver metabolomic changes in response to realgar administration (arsenic sulphide based traditional Chinese medication) and found decreased urinary levels of trimethylamine-N-oxide, phenylacetylglycine and hippurate and signs of impairment of amino acid metabolism.

Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR) combined with chemometric techniques is a powerful tool for urinary metabolomics and biomarker identification. A ^{1}H-

NMR profile of urine represents a snapshot into the biochemistry of a bio-fluid of an individual. It provides simultaneous qualitative and quantitative information on proteins and metabolites (such as carboxylic acids, ketones, glutamines) circulating in the body in response to factors such as diet, disease, ethnicity and gender (Holmes *et al.*, 2008, Psihogios *et al.*, 2008, Lenz *et al.*, 2004).

Studies on healthy populations have reported reference values for some urinary metabolites (Saude *et al.*, 2007). Furthermore, Ellis *et al.* (2010) applied ^{1}H-NMR Spectroscopy to investigate changes in urinary metabolomic profiles in response to cadmium exposure in residents at a contaminated site in Avonmouth (Bristol, United Kingdom), finding a significant correlation between urinary cadmium and 3-hydroxyisovalerate. Another study has been published on populations exposed to high levels of selenium in China (Zhu *et al*., 2002).

Despite the fact that urinary metabolomics by means of ^{1}H-NMR Spectroscopy is a promising tool for the study of body changes in relation to stress, disease and toxicity screening (Shockcor and Holmes, 2005), there are no reports of studies attempting to apply this technique to understanding the impact of arsenic exposure in humans.

This chapter is the first study that explores changes in urinary metabolomics in relation to arsenic and selenium urinary content in humans. Two cases of documented chronic arsenic exposure were selected. The first case was a UK resident population composed of Caucasians and UK-Bangladeshis, the latter with a reported arsenic exposure from rice (Chapter 3). The second case was a population resident in Bangladesh (from Araihazar), exposed to arsenic through drinking water (Ahsan *et al.*, 2006). Differences in urinary metabolites are discussed here and are explored in relation to ethnicity, migration, rice intake, and urinary arsenic and selenium. Urinary metabolites monitored in this chapter are detailed in Table A5-1 of the Appendix along with chemical structure and details on metabolic function.

2. Sampling populations

2.1 Arsenic exposure through rice consumption

A group of 33 Bangladeshis from the UK (UK-B), for which arsenic exposure from dietary route, (and especially rice) has been demonstrated previously (Chapter 3), was monitored.

The average UK Bangladeshi rice consumer ingested 21-fold more iAs from rice than a UK Caucasian, exceeding the WHO recommend maximum daily intake. Along with the UK B group, a small group of white Caucasians from the UK (UK-C, *n*=10) was also monitored. For both groups, the urinary arsenic profile and total selenium were reported elsewhere (Chapters 3 and 4). Both the UK-B and UK-C groups lived in Leicester or in London. Age and demographic characteristics of this study population are shown in Table 5-1. Smokers were excluded from the study to avoid the presence of an additive source of As. None of the Bangladeshi declared to consume alcohol, while all the Caucasians did. A significant increase in urinary DMA and iAs in UK Bangladeshis compared to UK Caucasians has been demonstrated in Chapter 3 as well as a positive significant correlation of this metabolite in urine with the daily rice intake in the study group.

Table 5-1: Basic characteristics of the UK resident population studied

	UK Bangladeshis	**UK Caucasians**
Characteristic		
Male (*n*)	22	3
Female (*n*)	11	7
Cigarette smoking	0	0
Age, Mean (SD)	47 (15)	32 (9)
BMI, Mean (SD)	26.7 (9.9)	22.1 (8.7)

2.2 Arsenic exposure from drinking water

The second study population was from Araihazar in Bangladesh (BD-B). This population was part of the Health Effects of Arsenic Longitudinal Study (HEALS) undertaken by Prof. Ahsan's group (Ahsan *et al.*, 2006). In the original project, a total of 11,746 men and women between 18 and 75 years were recruited. Participants completed a clinical evaluation, provided blood and spot urine samples. About one-third of the participants consumed water from a well with As concentration in each of three groups: >100 µg/L, 25-100 µg/L, and <25 µg/L.

A subset of 141 volunteers (demographics as in Table 5-2) were selected for this pilot study to explore the relationship between urinary metabolomics and arsenic exposure. Two aliquots of 2 ml of urine for each volunteer were transported in dry-ice to De Montfort University from the University of Chicago, and kept at -80°C till the day of the analysis. Urinary arsenic and selenium were performed on one of the aliquots by ICP-MS

(Essam Tahla's data). Metabolomics analysis on the other aliquot was performed by means of ^{1}H-NMR Spectroscopy at the University of Leicester.

Table 5-2: Demographics of participants from Bangladesh in this study

Characteristic	N	%
Sex		
Male	23	16.31
Female	118	83.69
Cigarette smoking		
Never	124	87.94
Former	7	4.96
Current	10	7.09
Age, Mean (SD)	36.7	9.9
BMI, Mean (SD)	20.2	3.2

3. Ethical approval

Ethical approval for the UK sampling activity and study was obtained from the De Montfort University ethics committee. For the study carried out in Bangladesh, ethical approval was gained from the University of Chicago and further approved by De Montfort University ethics committee for the samples to be received from USA and analysed in Leicester.

4. Materials

Deuterium oxide (D2O), 99.9 atom % D, containing 0.75 wt. % 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid, sodium salt (TSP) and standard series 5 mm NMR tubes, 400 mHz (Norell) were purchased from Sigma-Aldrich.

An Avance III AV- 500 spectrometer (Bruker) equipped with TOPSPIN 2.1 software was used for analysis and initial processing. The software Chenomx NMR Suite 7.0 (Chenomx Inc., Edmonton, Canada), was used for spectral pre-processing and metabolite profiling. Identification of peaks and quantification was achieved using the 500-MHz library from Chenomx NMR Suite 7.0, which uses the concentration of a known reference signal (in this case TSP) to determine the concentration of individual compounds.

To the best knowledge of the author, a urine reference material for urine metabolomics study has not been developed as yet. Therefore, certified reference materials developed

for arsenic and trace elements were used: NIES CRM n. 18 human urine and Trace elements in urine (lot 0511454, Seronorm), both were reconstituted in ultra-pure water as recommended by the producers. For statistical analysis PASW Statistics 18 (IBM) and SIMCA P+ 12.0 (Umetrics, Umeå, Sweden) were used.

5. Methods

Urine samples were prepared as mixtures of 90% Urine and 10% D_2O + 0.75% TSP, and immediately transferred into NMR tubes. All ^{1}H-NMR spectra were measured at 298K on a Bruker Avance III AV- 500 spectrometer using a 5mm BBO probe incorporating z-gradients, operating at 500.13 MHz for ^{1}H. Blanks and certified reference material were analysed along with urine samples. All spectra were acquired using the spectrometer TOPSPIN 2.1 software. Spectra were acquired with 64 transients each, using a standard 90^o pulse (10.25 µs) and a sweep width of 7500 Hz, accumulated with 64K data points (to produce a 4.3 s acquisition time). An additional recycle delay of 2 s gave an overall recycle time of 6.3 s between pulses. Data were processed using an exponential multiplication factor of 0.3 Hz. All chemical shift values were reported in parts per million (δ/ppm) and referenced to TSP (0 ppm). A flow chart of the steps from urine preparation to statistical classification used in this study is shown in Figure 5-1.

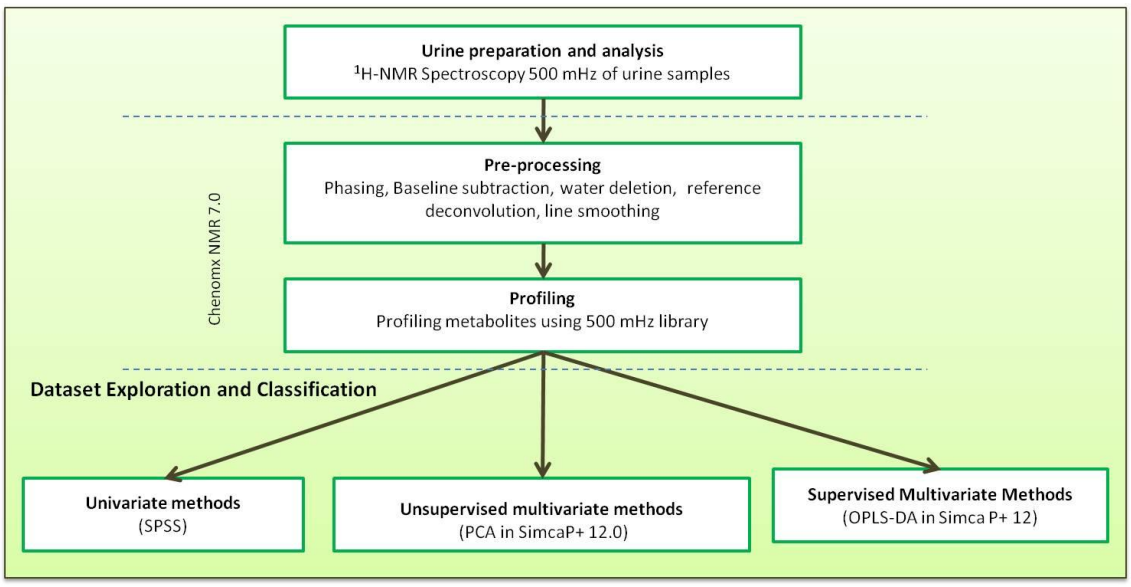


Figure 5-1: Flow chart of steps involved in urinary metabolomics profiling from preparation, through data treatment to statistical classification.

Pre-processing and target profiling were implemented in Chenomx. Metabolites were identified and quantified using the Profiler and Library Manager modules according to known chemical shift values. In order to statistically compare variables, concentrations have been exported as mg/dL and mM.

6. Statistics

Univariate and multivariate statistics were used in this study to extrapolate differences between groups. Both absolute (as mM and mg/dL) and relative (% metabolites) concentrations were used.

To calculate percentage metabolites, concentrations expressed as mg/dL, multiplied by dilution factor and normalised using the formula in Equation 7 were used.

$$\%Met_n = 100 * [Met_i] / \Sigma Met \qquad \text{(Equation7)}$$

$\%\ Met_n$: percentage normalised concentration of the metabolite
Met_i: concentration of the *i*-esim metabolite [mg/dL]
Σ Met: summatory of the metabolites in a urine sample [mg/dL]

7. Results

Table 5-3 shows the full list of profiled metabolites for the UK residents (UK) and Araihazar residents (BD). A typical urine NMR spectrum in the region between 0 and about 4.1 ppm is shown in Figure 5-2, highlighting peaks belonging to the main metabolites.

Examples of selected, full NMR spectra are reported in the Appendix to this chapter in Figure A5-1 and A5-2.

Table 5-3: List of profiled metabolites derived from the urine ^{1}H NMR spectra of the study population from Bangladesh (BD) and the United Kingdom (UK)

	Metabolite	Study population		Metabolite	Study population
1	2-Hydroxyisobutyrate	UK + BD	**15**	Dimethylamine	UK + BD
2	2-Hydroxyisovalerate	UK	**16**	Formate	UK + BD
3	3-Aminoisobutyrate	UK	**17**	Glycine	UK + BD
4	3-Hydroxybutyrate	UK	**18**	Hippurate	UK + BD
5	3-Hydroxyisovalerate	UK + BD	**19**	Lactate	UK + BD
6	Acetate	UK + BD	**20**	*N,N*-Dimethylglycine	UK + BD
7	Acetoacetate	UK + BD	**21**	*N*-Acetylglycine	UK
8	Acetone	UK + BD	**22**	*N*-Phenylacetylglycine	UK
9	Alanine	UK + BD	**23**	Pyruvate	UK + BD
10	Betaine	UK + BD	**24**	Succinate	UK + BD
11	Citrate	UK + BD	**25**	Taurine	UK + BD
12	Creatine	UK + BD	**26**	Threonine	UK + BD
13	Creatinine	UK + BD	**27**	Trimethylamine	UK + BD
14	*cis*-Aconitate	UK	**28**	Trimethylamine N-oxide	UK + BD
			29	*trans*-Aconitate	UK

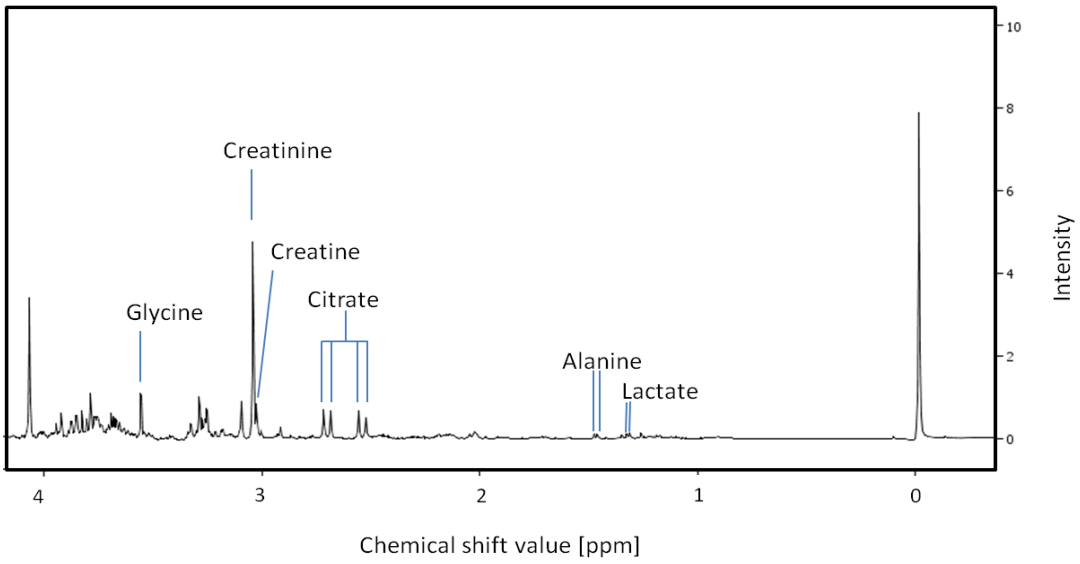


Figure 5-2: Typical urinary ^{1}H-NMR spectra in the region between 0 and 4.2 ppm, peaks corresponding to some profiled metabolites are highlighted.

7.1 Urinary metabolomic profiles of the UK residents

The most abundant compound of those determined in urine of the overall UK population (43 volunteers) was creatinine, which accounted for 46 ± 11 % of the profiled compounds, followed by citrate and hippurate as reported in Table 5-4.

Relative % of some metabolites were normally distributed (such as alanine, creatinine) while others were not (i.e. betaine, dimethylamine). Two typical ^{1}H NMR spectra of urine for a UK-B and a UK-C are shown in Figure 5-3, along with pie charts for urinary % composition for the selected UK-Bangladeshis and Caucasian volunteers.

Table 5-4: Abundance of % metabolites of profiled metabolites in urine of 43 volunteers from the UK and the results of normality test

Metabolite	Average	SD	Normality test*
Creatinine	45.95	± 10.96	passed
Citrate	14.17	± 6.69	passed
Hippurate	10.08	± 7.96	failed
Glycine	4.84	± 3.75	failed
N-Phenylacetylglycine	3.47	± 2.42	passed
Creatine	3.47	± 3.42	failed
cis-Aconitate	3.17	± 2.00	failed
Taurine	2.95	± 2.71	failed
Trimethylamine N-oxide	1.62	± 3.84	failed
Betaine	1.27	± 1.68	failed
Alanine	1.24	± 0.66	passed
Lactate	1.12	± 2.07	failed
trans-Aconitate	0.91	± 0.69	failed
N-Acetylglycine	0.86	± 0.61	failed
3-Aminoisobutyrate	0.84	± 1.23	failed
Dimethylamine	0.66	± 0.24	failed
Formate	0.61	± 0.75	failed
Acetoacetate	0.49	± 0.53	failed
Threonine	0.34	± 0.23	passed
N,N-Dimethylglycine	0.31	± 0.18	passed
Succinate	0.29	± 0.28	failed
3-Hydroxyisovalerate	0.27	± 0.11	passed
3-Hydroxybutyrate	0.24	± 0.20	failed
2-Hydroxyisobutyrate	0.22	± 0.09	passed
Acetate	0.21	± 0.15	failed
Pyruvate	0.16	± 0.18	failed
Acetone	0.12	± 0.20	failed
Trimethylamine	0.11	± 0.21	failed
2-Hydroxyisovalerate	0.04	± 0.08	failed

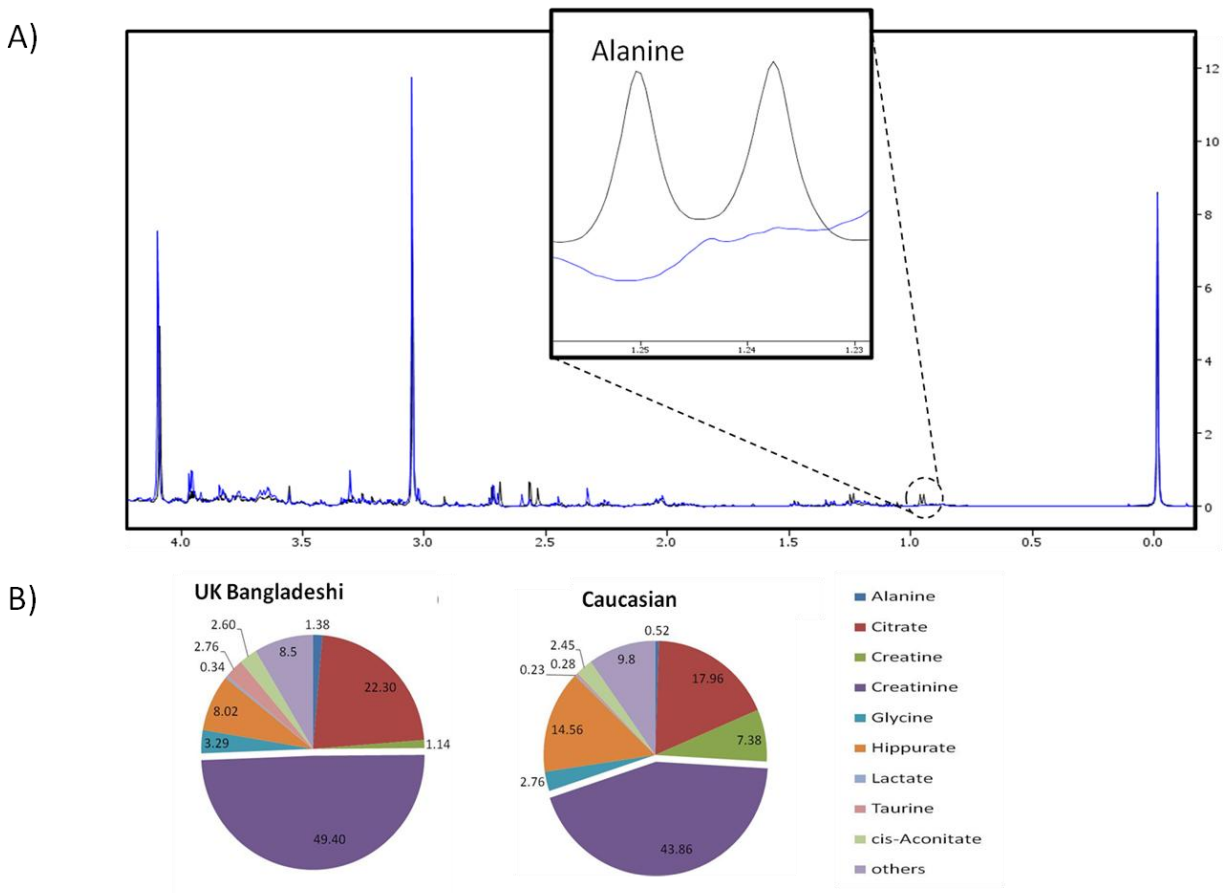


Figure 5-3: (A) ^{1}H-NMR spectra for urine of a UK Bangladeshi (black line) and a Caucasian (blue line) with the inset focusing on the alanine doublet peaks. (B) Urinary % composition for some of the profiled metabolites in a UK Bangladeshi and a Caucasian volunteer: in each case, creatinine represents the greatest proportion of the metabolites analysed in the urine samples using ^{1}H NMR spectroscopy.

Differences in relation to ethnicity between the UK-B and UK-C urine were further explored. Univariate descriptive statistics on %metabolites showed significant differences as highlighted in Table 5-5. Parametric or non parametric tests were run to assess these differences. The UK-B group showed a particularly pronounced increase in %N,N-dimethylglycine (%DMG) and %alanine compared to UK-C. Furthermore, %betaine, %taurine and %glycine were also significantly higher for the UK-B group compared to the UK-C. On the contrary, UK-B had lower %hippurate and %succinate compared to the Caucasians. Box plots for a selection of significant different features are presented in Figure 5-4.

Table 5-5: Descriptive statistics and inter-ethnicity comparison for significant urinary metabolites in Bangladeshi and Caucasians in the UK

% Metabolite[a]	UK- Bangladeshis	UK-Caucasians	P [b]	OPLS-DA[c]
Creatine	2.14 (0.09-14.82)	3.29 (1.03-13.34)		↓
Betaine	0.91 (0.24-9.71)	0.38 (0.16-1.10)	0.002[m]	↑
***N,N*-Dimethylglycine**	0.37 ± 0.03	0.13 ± 0.02	<0.001	↑
Alanine	1.43 ± 0.11	0.61 ± 0.09	<0.001	↑
Taurine	3.34 ± 0.51	1.69 ± 0.36	0.038[m]	↑
Glycine	4.1 (1.4-18.6)	2.61 (1.01-4.83)	0.021[m]	↑
Succinate	0.17 (<LOD-1.48)	0.37 (0.19-1.00)	0.002[m]	
Hippurate	6.99 (0.41-44.8)	13.89 (9.40-20.57)	0.003[m]	↓

(a)Urinary concentrations are expressed as mean concentration ± standard error of the % metabolites normally distributed (alanine and N,N-dimethylglycine). For all the other variables medians are reported followed by min-max in parentheses; (b) P values derived from the t-test when the values are normally distributed or Mann-Whitney ([m]) if otherwise. (c) Important features increased (↑) or decreased (↓) in UK-B identified by OPLS-DA supervised multivariate approach.

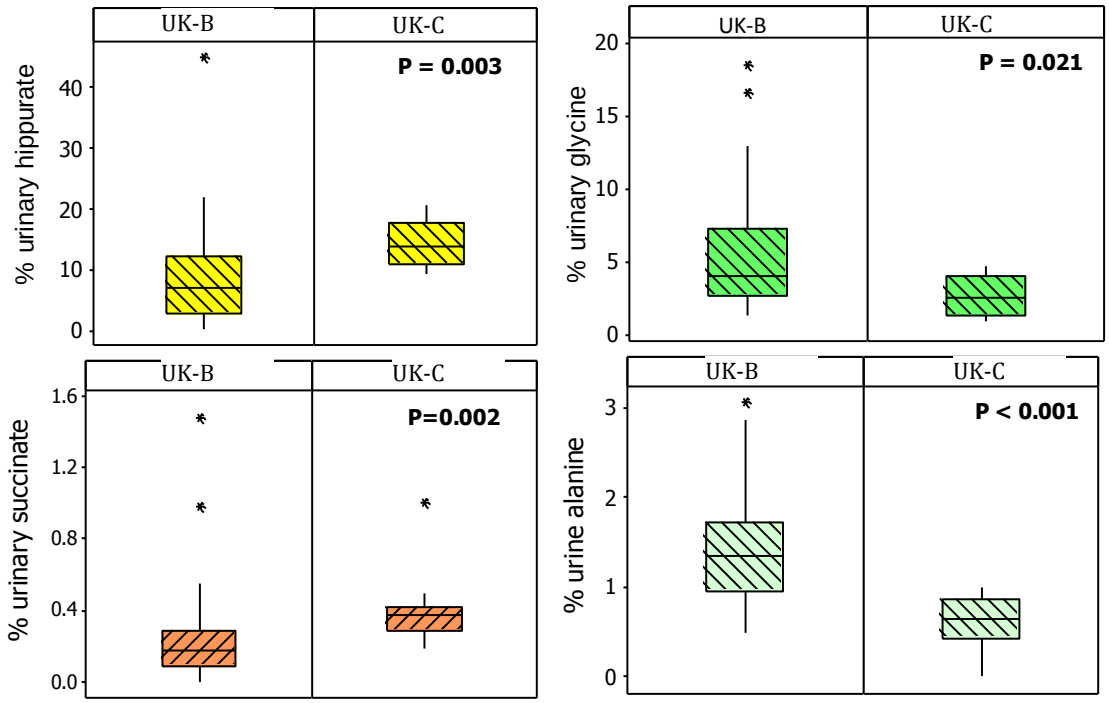


Figure 5-4: Box plots of urinary %hippurate, %succinate, %glycine and %alanine for UK Bangladeshi (UK-B) and Caucasians (UK-C), stars represent outliers.

When exploring the dataset by multivariate statistics, Principal Component Analysis (PCA) was applied in order to investigate the presence of differences related to ethnicity. Some separation was seen on the second component (see Figure 5-5 A) between UK-B and UK-C. In order to identify which factors drive this separation, a supervised method was used. An OPLS-DA analysis was performed (R^2= 0.3; R^2Y: 0.8 and Q^2= 0.3) allowing a clearer separation between the UK-B group and UK-C to emerge, as shown in Figure 5-5 B. The main metabolites driving this difference are shown in Fig 5-6 A. Figure 5-6 B focuses on %alanine. The results are in agreement with the univariate approach for urinary %alanine, %betaine, %*N,N*-dimethylglycine, %taurine, %glycine and %hippurate. Important features identified by OPLS-DA are: a decrease in %creatine and increase in %trimethylamine and % *trans*-aconitate for UK Bangladeshis compared with UK-Caucasians.

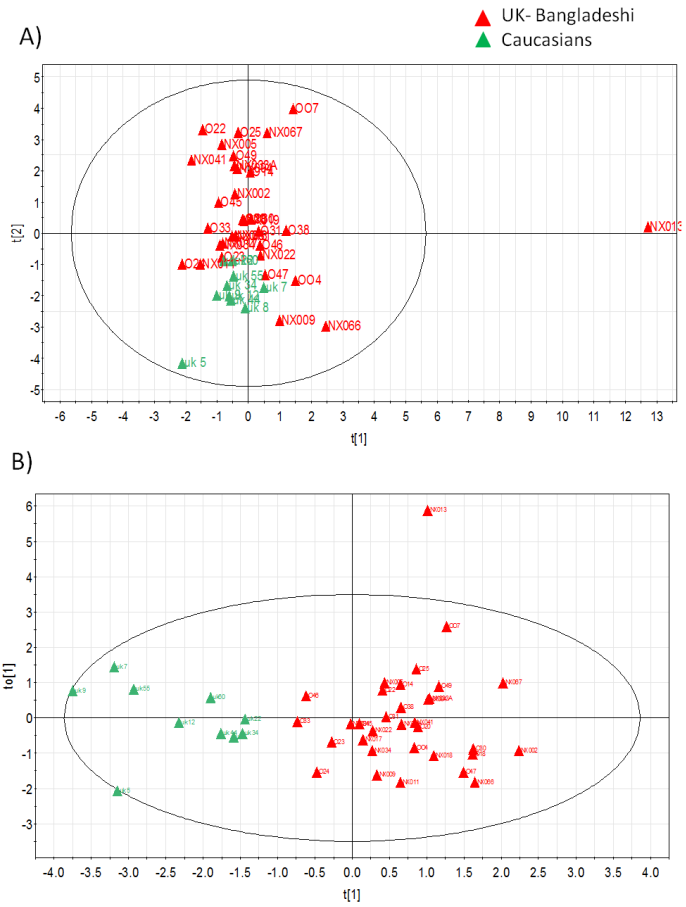


Figure 5-5: Multivariate analysis performed on urinary metabolomic profile of the UK-Bangladeshi and Caucasians: A) PCA denotes a certain extent of separation of UK Bangladeshi and Caucasians; B) OPLS-DA model performed using ethnicity as Y in the form of a binary vector allows one to attain a greater separation between UK-B and UK-C.

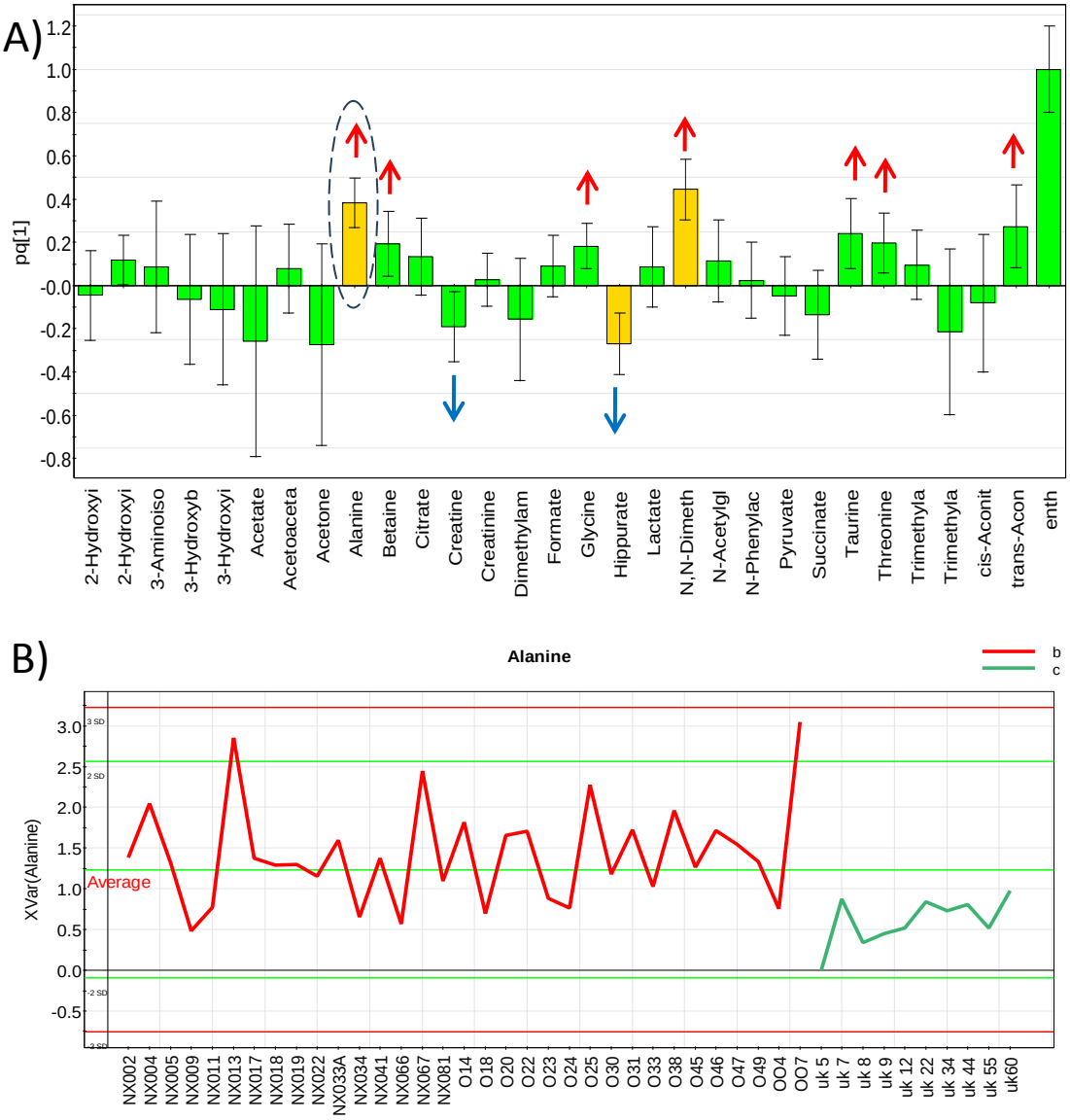


Figure 5-6: (A): Important features driving separation of UK Bangladeshi and UK-Caucasians. Red arrows indicate metabolites that have increased in urine of the UK-B and blue arrows metabolites that have decreased in UK-B compared to UK-C; (B): focus on %alanine: increase in %alanine in UK Bangladeshis (b, red line) in comparison to UK Caucasians (c, green line). In the plot average, 2 standard deviations (green horizontal) and 3 standard deviations (red horizontal) are shown.

7.2 Factors affecting urinary metabolomic profile within the UK group

Thanks to the use of a questionnaire, information on the general habits and diet of the two groups were collected. An attempt to rationalise the findings in terms of %metabolites differences between UK-C and UK-B was made in light of the acquired information.

7.2.1. Chewing Pan

Sixteen volunteers within the UK-B group were found to chew areca nut with or without tobacco. A Mann-Whitney test was used to compare urinary %metabolites of chewers and non chewers: the only affected metabolite was 3-hydroxisovalerate, which was significantly lower in chewers (p=0.028) than in non-chewers.

7.2.2. Gender

Gender difference was explored by means of Mann-Whitney test within the UK-B group and the only significant difference was found in urinary %creatine (p=0.002), which was higher in females than in males.

7.2.3 Rice

Within the overall UK residents, 39 volunteers gave details for rice consumption and could be included in the analysis. The impact of rice consumption was explored in the overall UK population by means of the Pearson correlation test between the log concentration of rice intake [g/day] and urinary metabolites.

As reported in Table 5-6, significant positive correlations were found between rice intake and %alanine (p=0.001), %N,N-dymethylglycine (p<0.001) and %taurine (0.020). Conversely, rice intake was negatively correlated to %succinate (p=0.003), %creatine (0.048), %acetate (0.025). A trend of decrease was also seen for %hippurate (p=0.054).

Table 5-6: Correlation between rice intake log [g/day] and % urinary metabolites

Metabolite%	Pearson correlations	P	OPLS-DA
Alanine	.518	0.001	↑
N,N-Dimethylglycine	.552	<0.001	↑
Acetate	-.358	0.025	
Creatine	-.318	0.048	
Hippurate	-.311	0.054	↓
Succinate	-.465	0.003	↓
Taurine	.370	0.020	↑

OPLS-DA indicates features that increase (↑) or decrease (↓) in relation to rice consumption according to supervised multivariate approach.

Scatter plots are reported in Figure 5-7 from some metabolites. Using a multivariate approach (OPLS-DA), a fairly good model (R^2Y: 0.8, Q^2: 0.4) was obtained using rice intake (log scale) as the Y variable. In Figure 5-8, the metabolites driving the separation are represented. Increases in %alanine, %glycine, %*N,N*-dimethylglycine, %taurine and %trans-aconitate are positively correlated to higher rice intake. In contrast, rice consumption was correlated with a decrease in %hippurate and %succinate in urine of this study group.

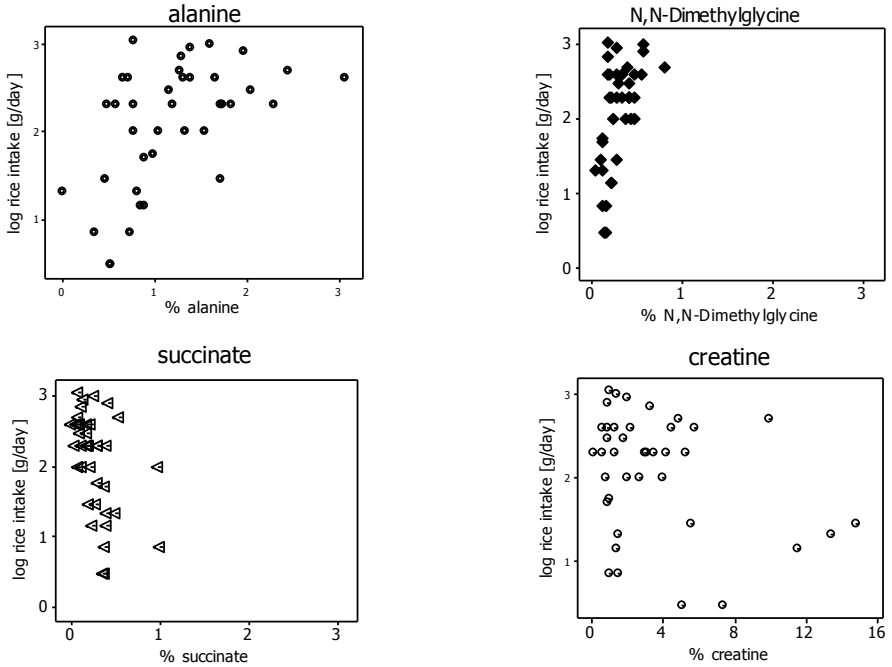


Figure 5-7: Relationship between rice intake (expressed as g/day on a log scale) and urinary %alanine, %*N,N*-dimethylglycine, %succinate and %creatine in 39 volunteers from the UK study group who provided information on rice consumption habits.

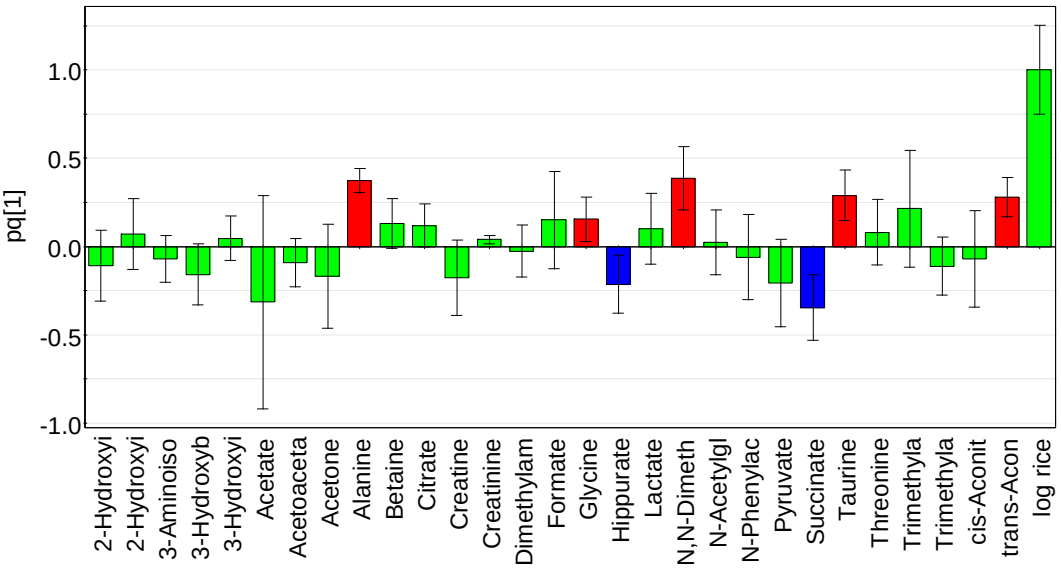


Figure 5-8: Important features identified by OPLS-DA and correlated to log rice consumption in the UK study population.

7.2.4. Diabetes

Eleven subjects within the UK Bangladeshi group reported being affected by diabetes.

For most of the urinary profiled features, the diabetic group in this study did not differ significantly from the non-diabetic group except for %betaine (on a Mann-Whitney test, P= 0.002), %alanine (P=0.009), %*N,N*-dymethylglycine (P=0.005), all of which were higher in the diabetic group compared to the non-diabetic irrespective of ethnicity. Box plots for the three groups are in Figure 5-9.

In order to investigate if this difference was driving the inter-ethnicity separation between UK-Caucasians and UK-Bangladeshis, a Mann-Whitney test was preformed excluding the diabetic subjects, and therefore comparing 10 Caucasians and 22 Bangladeshis. The important features responsible for the B and C separation were still significant even when excluding the diabetic group, especially: % urinary alanine (p = 0.001), which was higher in non-diabetic Bangladeshis (median 1.3%) compared with Caucasians (0.6%); betaine (P=0.009), which was higher in the Bangladeshis (median 0.7 %) versus Caucasians (0.4%); *N,N*-dimethylglycine (P=0.001), which was 0.3% versus 0.1% in B and C respectively. No significant difference was seen for the other metabolites in relation to diabetes.

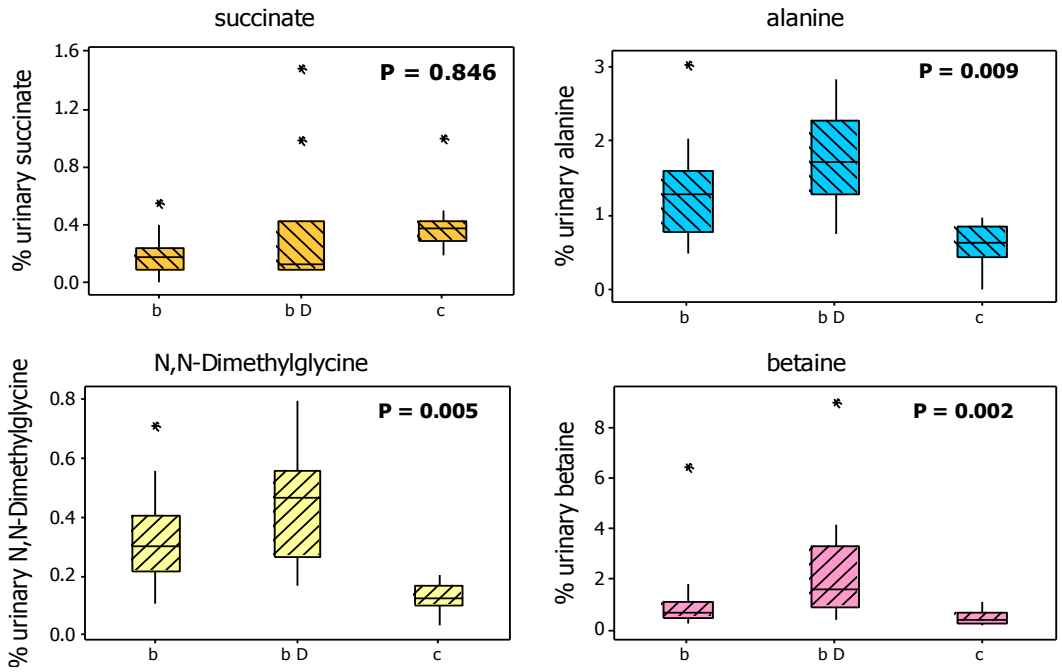


Figure 5-9: Urinary metabolites for the UK study group divided in non diabetic Bangladeshi (b), diabetic Bangladeshi (b D) and Caucasians (c).

7.2.5. Arsenic and Selenium

Results from urinary arsenic analysis for the UK resident group (results shown in Chapter 3), were used to correlate total arsenic, percentage of dimethylarsinic acid on total arsenic (%DMA), percentage of monomethylarsonic acid (%MA) and percentage of inorganic arsenic (%iAs) to urinary metabolites profiled.

A Spearman test considering total arsenic, %DMA, %MA and %iAs in urine versus each % metabolite in the overall UK population revealed some significant relationships as shown in Table 5-7.

The most significant positive correlation was seen between %DMA and %*N,N*-dimethylglycine. Additionally, %DMA significantly positively correlated to %alanine, %betaine and negatively to %hippurate. A negative trend was seen for %succinate. Urinary %MMA was positively correlated to %*N*-phenylacetylglycine. %iAs was significantly negatively correlated to %pyruvate. The relationship between %*N,N*-dimethylglycine and %DMA in urine is reported in Figure 5-10.

Table 5-7: Significant features identified from Spearman test in the UK study group (*n*=43)

Y	X	Correlation Coefficient	P
As	% creatinine	0.352	0.021
% DMA	%alanine	0.387	0.010
% DMA	%DMG	0.520	<0.001
% DMA	%hippurate	-0.371	0.014
% DMA	%betaine	0.319	0.037
% DMA	%succinate	-0.291	0.058
% MMA	%N-Phenylacetylglycine	0.305	0.047
% iAs	%pyruvate	-0.328	0.032
Se	% creatinine	0.431	0.004
Se	% betaine	-0.330	0.031
Se	%dimethylammine	0.377	0.013

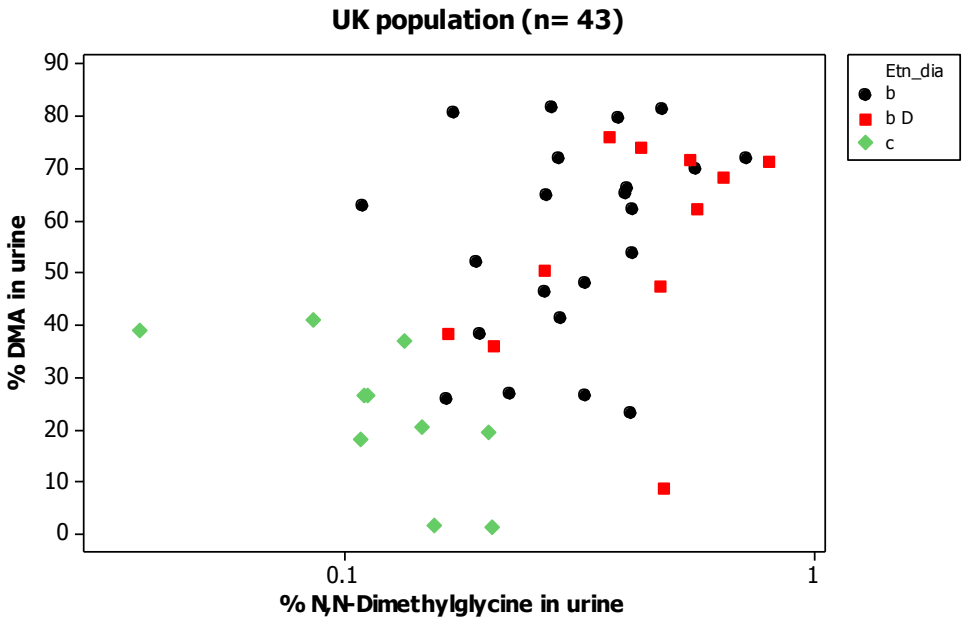


Figure 5-10: a significant relationship was observed between %DMA and %N,N-dymethylglycine in urine of UK volunteers. Black dots represent Bangladeshis that did not declare to be affected by diabetes, red squares represent Bangladeshis who declared to be affected by diabetes and green diamonds represent UK-Caucasians.

A OPLS-DA model using %DMA as Y, (R^2Y= 0.5 and $Q^2Y < 0$) was fit. Even though the model was not predictive, features having the greatest impact were %alanine, %*N,N*-dimethylglycine and %hippurate, which confirms the results from the univariate approach.

Total urinary selenium (corrected using SG) was positively correlated to %creatinine (r=0.431, P = 0.004) and %dimethylammine (r =0.337, P = 0.013) and negatively (r =-0.330, P = 0.031) to %betaine.

7.3 Volunteers from Bangladesh

When treating and profiling spectra, a marked reduction in the concentration of the metabolites for BD-B compared to UK residents was noticed. Indeed, the NMR peaks were so weak for the BD-B group that profiling was only possible for a smaller number of metabolites (see Figure 5-11 and Table 5-3).

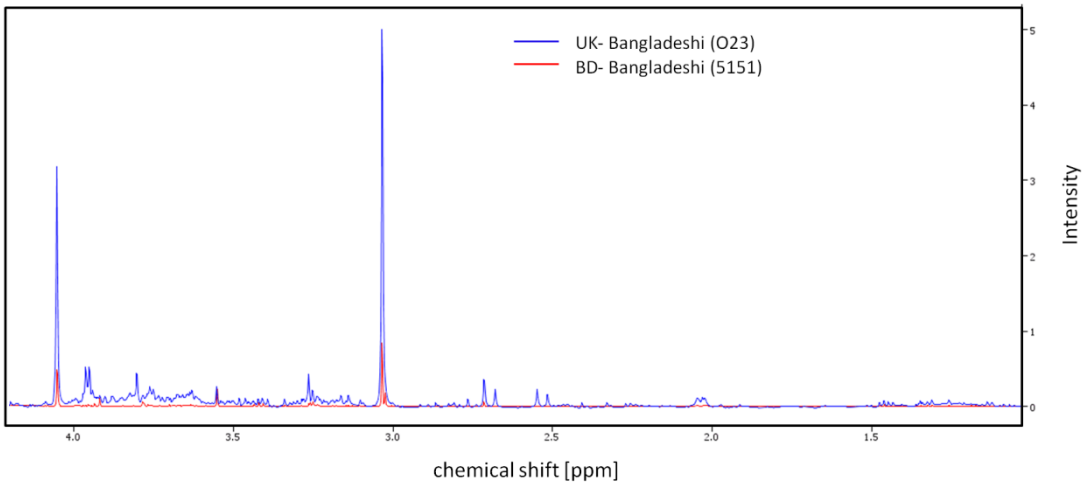


Figure 5-11: Comparison of urinary spectra for a UK Bangladeshi volunteer (blue line) and a BD Bangladeshi (red line), note sensible reduction of peak height in UK-Bangladeshi samples caused by lower metabolite concentrations.

Most of the urinary metabolite concentrations were lower in the BD-B group than in the UK-B group. The sum of commonly profiled metabolites was highly significantly lower in BD-B (74 ± 49 mg/dL) than in UK-B (155 ± 80 mg/dL) with P<< 0.001. Furthermore, to corroborate this observation, specific gravity (SG) was compared; SG was significantly lower in urine of the BD-B group (1.009±0.006) than the UK-B group (1.014±0.005) (p<<0.001).

A comparison of UK-B versus BD-B by means of logistic regression is given in Table 5-8. A general significant reduction in urinary metabolites is evident since all beta coefficients are negative. The most significant reductions in concentration were seen for 3-hydroxyisovalerate, creatine and creatinine. Reductions were also evident for 2-

hydroxyisobutyrate, acetoacetate acetone, alanine, betaine, citrate, formate, glycine, lactate, *N,N*-dimethylglycine, succinate, taurine and trimethylamine. Furthermore, some metabolites were not affected in terms of absolute concentration by migration, including acetate, pyruvate and threonine. The concentrations of these metabolites in the Bangladeshis from Araihazar were comparable to the ones from UK Bangladeshis.

Table 5-8: Comparison of UK-Bangladeshi versus BD-Bangladeshi, [mg/dL], significant features are in bold

	Beta*	SE	P value
2-Hydroxyisobutyrate	**-0.727**	**0.301**	**0.017**
3-Hydroxyisovalerate	**-1.440**	**0.344**	**<.0001**
Acetate	-0.222	0.427	0.604
Acetoacetate	**-0.987**	**0.441**	**0.027**
Acetone	**-1.108**	**0.404**	**0.007**
Alanine	**-0.685**	**0.281**	**0.016**
Betaine	**-0.806**	**0.357**	**0.025**
Citrate	**-1.635**	**0.570**	**0.005**
Creatine	**-1.753**	**0.451**	**<.0001**
Creatinine	**-0.850**	**0.217**	**<.0001**
Dimethylamine	**-0.605**	**0.220**	**0.007**
Formate	-1.252	0.351	**0.001**
Glycine	**-0.693**	**0.316**	**0.030**
Hippurate	-0.736	0.424	0.085
Lactate	**-0.987**	**0.370**	**0.008**
N,N_Dimethylglycine	**-1.285**	**0.402**	**0.002**
Pyruvate	-0.416	0.553	0.453
Succinate	**-1.275**	**0.463**	**0.007**
Taurine	**-1.055**	**0.381**	**0.006**
Threonine	-0.338	0.414	0.416
Trimethylamine	**-0.804**	**0.384**	**0.038**
TMAO	-0.611	0.350	0.083

Data adjusted for sex, age, BMI, As, and Se; *Beta estimated for BD-B compared to UK Bangladeshi, SE: standard error.

A comparison to published literature was made on the basis of absolute concentrations (expressed as mM). Urinary metabolite concentrations for the overall UK population (*n*= 43) were in good agreement to literature references as in Table 5-9.

Figure 5-12 shows a comparison, based on median values, between the three study groups and values reported in the literature. For some metabolites, such as 2-hydroxisobutyrate,

3-hydroxisovalerate, succinate, lactate, dimethylamine, creatinine (Figure 5-5 b) and acetoacetate it was DB-B < UK-B < UK-C. The reduction in 3-hydroxyisovalerate in DB-B compared to both literature and the other study groups was quite pronounced. A second group of metabolites showed a comparable concentration in urine of BD-B and UK-B, and both were lower than UK-C, as for pyruvate, creatine, acetate and hippurate. Some metabolites were similar in the three groups: threonine, alanine, taurine, glycine. UK-B showed higher levels of betaine and N,N-dymethylglycine than the other groups. Finally, for citrate, UK-C and UK-B had similar values while BD-B had a lower concentration in urine (see Figure 5-12 and 5-13-a).

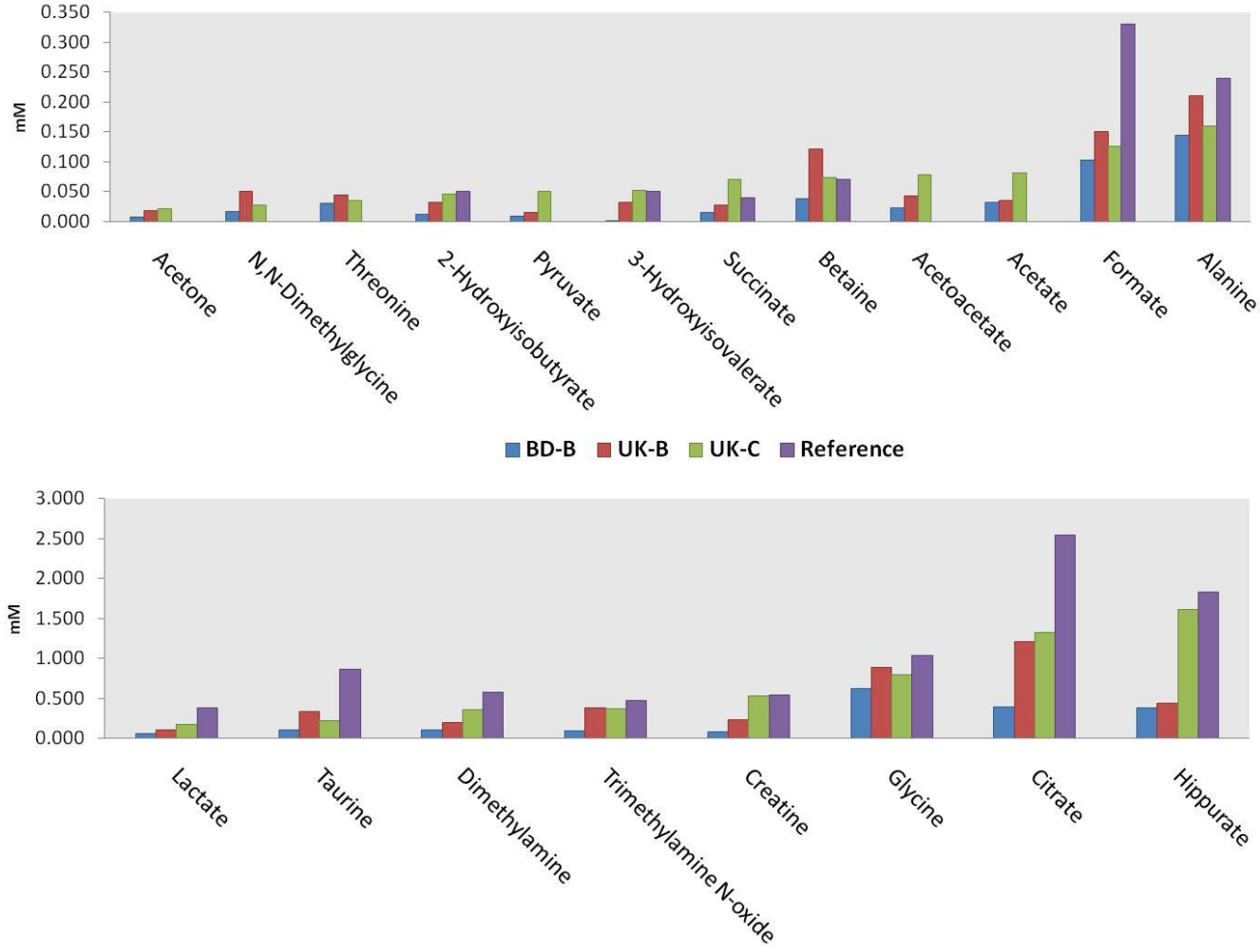


Figure 5-12: Comparison of urinary metabolites for BD-Bangladeshis, UK-Bangladeshis, UK Caucasians from this study and reference values as reported in Table 5-10. Bars are based on medians.

Table 5-9: Urinary metabolites (mean concentrations, mM) standard deviation (SD), 5th percentile (5th) and 95th percentile in urine of BD-Bangladeshis, UK-Bangladeshis and UK-Caucasians. References: (1) (Saude et al., 2007); (2) (Shaykhutdinov et al., 2009).

	BD-Bangladeshis (*n*=141)					UK Caucasians (n=10					UK –Bangladeshis (n=33)					Reference		
Metabolite	**mean**	**SD**	**median**	**5th**	**95th**	**mean**	**SD**	**median**	**5th**	**95th**	**mean**	**SD**	**median**	**5th**	**95th**	**mean**	**SD**	
Acetone	0.012	0.024	0.007	<LOD	0.037	0.131	0.308	0.022	0.013	0.605	0.023	0.027	0.018	0.006	0.042			
***N,N*-Dimethylglycine**	0.027	0.037	0.017	<LOD	0.083	0.031	0.019	0.028	0.007	0.061	0.058	0.040	0.050	0.019	0.143			
Threonine	0.051	0.061	0.030	0.005	0.177	0.050	0.038	0.034	0.014	0.110	0.056	0.056	0.045	0.005	0.144			
2- Hydroxyisobutyrate	0.013	0.013	0.011	<LOD	0.038	0.058	0.038	0.046	0.015	0.118	0.035	0.024	0.031	0.009	0.076	0.05	0.03	2
Pyruvate	0.017	0.040	0.008	<LOD	0.041	0.056	0.034	0.051	0.016	0.109	0.025	0.026	0.014	0.001	0.068			
3-Hydroxyisovalerate	0.008	0.013	<LOD	<LOD	0.038	0.069	0.062	0.052	0.012	0.171	0.039	0.028	0.032	0.011	0.096	0.050		1
Succinate	0.020	0.025	0.014	0.001	0.066	0.086	0.068	0.070	0.023	0.195	0.031	0.031	0.027	0.004	0.066	0.039	0.002	1
Betaine	0.061	0.084	0.038	0.008	0.172	0.081	0.041	0.074	0.029	0.131	0.230	0.380	0.121	0.041	0.660	0.071	0.026	1
Acetoacetate	0.045	0.065	0.023	<LOD	0.135	0.105	0.079	0.079	0.028	0.239	0.071	0.087	0.043	0.010	0.246			
Acetate	0.094	0.426	0.031	0.003	0.164	0.123	0.152	0.082	0.024	0.365	0.051	0.040	0.034	0.016	0.095			
Formate	0.152	0.135	0.102	0.014	0.406	0.225	0.295	0.125	0.018	0.724	0.225	0.288	0.151	0.031	0.519	0.33		
Alanine	0.175	0.128	0.144	0.036	0.374	0.161	0.107	0.160	0.025	0.298	0.262	0.148	0.211	0.079	0.501	0.24		
Lactate	0.122	0.439	0.066	0.011	0.217	0.182	0.143	0.175	0.033	0.401	0.182	0.203	0.107	0.015	0.579	0.38		
Taurine	0.155	0.182	0.109	<LOD	0.433	0.283	0.186	0.223	0.092	0.588	0.424	0.402	0.339	0.060	1.271	0.867	0.249	1
Dimethylamine	0.128	0.098	0.107	0.048	0.299	0.415	0.242	0.361	0.139	0.810	0.251	0.208	0.195	0.051	0.658	0.583	0.134	1
TMAO	0.118	0.104	0.093	0.017	0.301	1.282	2.972	0.371	0.099	5.612	0.219	0.195	0.386	0.029	0.664	0.475	0.087	1
Creatine	0.198	0.396	0.081	0.015	0.745	1.019	1.028	0.539	0.076	2.665	0.410	0.530	0.235	0.047	1.098	0.55	0.49	2
Glycine	0.923	0.867	0.620	0.143	2.521	0.990	0.819	0.798	0.218	2.384	1.293	1.285	0.892	0.258	3.497	1.037	0.113	1
Citrate	0.551	0.580	0.394	0.006	1.676	1.543	1.270	1.323	0.384	3.504	1.364	1.013	1.207	0.231	3.088	2.54		2
Hippurate	0.544	0.560	0.379	0.046	1.444	2.003	0.994	1.613	0.768	3.222	0.875	1.095	0.440	0.105	3.037	1.83		2
Creatinine	2.943	2.117	2.439	1.082	9.771	4.632	9.568	3.406	15.44	6.917	3.780	6.022	1.476	12.951	15.24	9.03	4.37	2

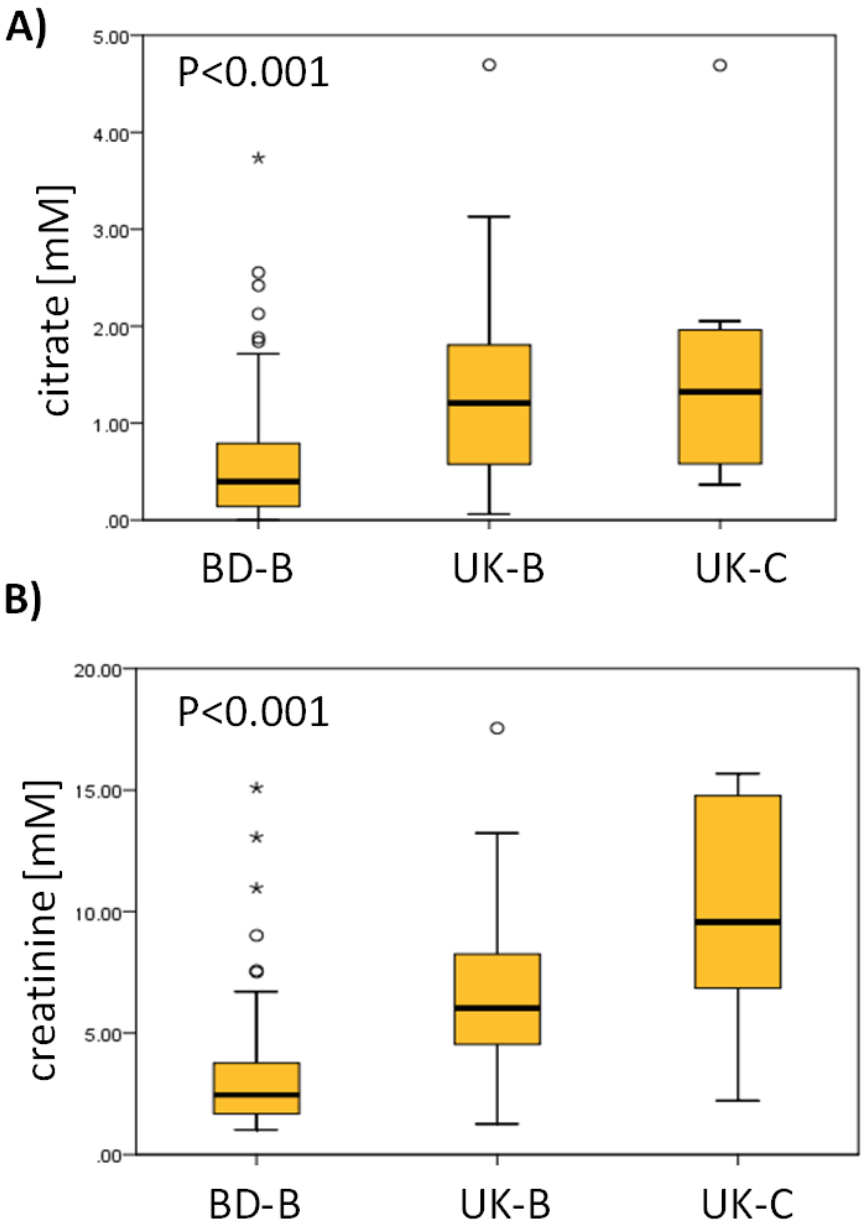


Figure 5-13: Comparison of concentration [mM] of main metabolites in urine of Bangladeshi from Bangladesh (BD-B) and the Bangladeshis residing in United Kingdom (UK-B) and Caucasians from the UK (UK-C); citrate (A)and creatinine (B).

7.3.1. The effect of migration on the Bangladeshi population

In order to appreciate differences in urinary metabolomic profile independently from glomerular filtration rate and to compare the two study populations from UK-B and DB-B in order to appreciate the effect of migration, % metabolites (as calculated using Equation 6) were considered.

An OPLS-DA analysis revealed differences in terms of percentage metabolites between the two groups. UK-Bangladeshis had a lower urinary %acetate, %alanine, %glycine, %threonine and %hippurate compared to BD-Bangladeshis. On the contrary %2-hydroxyisobutyrate, %3-hydroxyisovalerate, %citrate and %betaine were higher in UK-Bangladeshis compared to the BD-B group as shown in Figure 5-14 and summarised in Table 5-10.

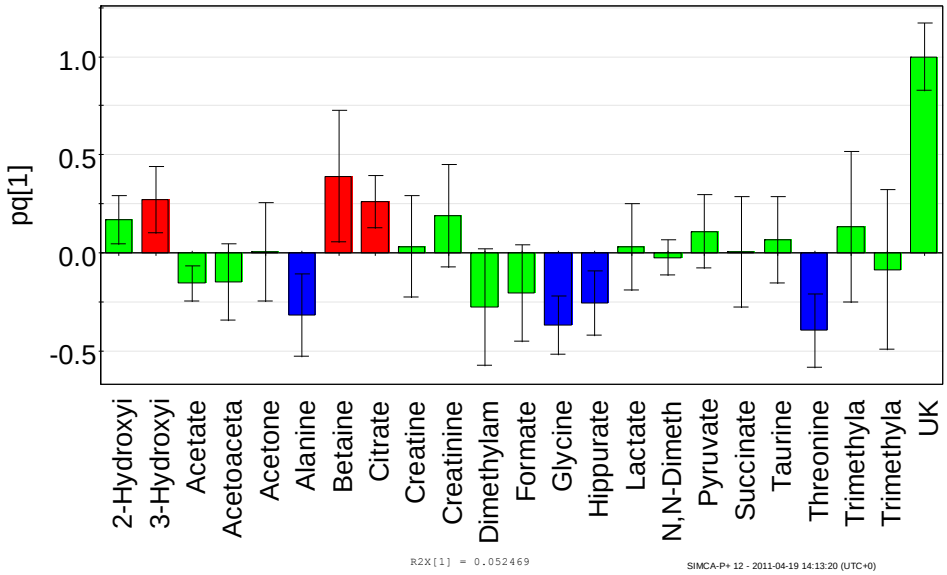


Figure 5-14: Important features driving separation of UK-Bangladeshis and BD-Bangladeshis recognised by OPLS-DA. Error bars represent Jacknife errors. UK=1 for UK-Bangladeshis.

Table 5-10: Important Features in driving the difference between UK B and BD-B

Metabolites	UK Bangladeshis
% 2-hydroxyisobutyrate	↑
% 3-hydroxyisovalerate	↑
% acetate	↓
% betaine	↑
% alanine	↑
% citrate	↑
% glycine	↓
% hippurate	↓
% threonine	↓

7.3.2. Arsenic and selenium

Univariate correlations in urine of BD-B volunteers from Araiazhar were investigated by means of a Spearman test. Total arsenic was significantly correlated to %dimethylamine (r =0.169, P=0.044) and marginally to %acetone (r= 0.148, P = 0.078). For total arsenic in urine of BD-B volunteers it was not possible to fit a good model by means of OPLS-DA.

For selenium, the Spearman test gave results as reported in Table 5-11.

Table 5-11 Significant features identified from Spearman test in the BD study group (*n*=141)

Y	X	Correlation coefficient	P value
Se	%alanine	-0.718	0.034
Se	%DMG	-0.195	0.020
Se	%3-hydroxyisovalerate	-0.218	0.009
Se	% creatinine	0.302	<0.001
Se	% dimethylamine	0.312	<0.001
Se	%glycine	-0.223	0.008
Se	%citrate	-0.252	0.002

%DMG = %*N*,*N*-dimethylglycine

Urinary selenium was significantly negatively correlated to %3-hydroxyisovalerate, %alanine, %glycine, %citrate. In contrast, selenium was positively correlated with %DMG %creatinine and %dimethylamine. An OPLS-DA model was fitted using Se as the Y variable, which despite the fact it shows low R^2 and Q^2 values, was able to identify %creatinine, %dimethylamine and %3-hydroxyisovalerate as the most important features.

8. Discussion

Urine is a complex mixture of several compounds and ^{1}H-NMR Spectroscopy offers the possibility to perform a qualitative and quantitative screening of human metabolites simultaneously in a non-invasive manner.

By means of ^{1}H-NMR Spectroscopy, the effect of arsenic and selenium exposure on the urinary metabolomic profile was explored in this chapter. Three study populations were monitored: UK-B, UK-C and BD-B. The effect of ethnicity, migration from Bangladesh to the UK, rice consumption, chewing pan and diabetes were investigated as well.

Within the UK residents, Bangladeshi and Caucasian urinary metabolomic profiles differed significantly, as determined by both univariate and multivariate approaches. The most pronounced difference was the significant increase of urinary %DMG and %alanine in UK-

Bangladeshis compared to UK-Caucasians. The UK-B group showed significantly higher %betaine, %glycine, %taurine, %threonine and % trans-aconitate than UK-C. In contrast, %creatine, %succinate and %hippurate were lower in urine of UK-B than in UK-C. There might be several different reasons for such differences in urinary metabolomic profile of these two ethnic groups living in the UK. A possible interpretation of the screened differences follows.

Diet is one of the main factors acting on urinary metabolomics and probably plays the main role in driving the separation of urinary metabolomic profiles of UK-B and UK-C. Holmes (Holmes *et al.*, 2008) demonstrated the presence of well defined metabolic phenotypes for 4,630 volunteers from UK, USA, China and Japan, discriminating West from East groups, with geographic metabolic differences playing a greater role than gender. The main discriminating features in the latter study were metabolites from dietary origin such as creatine, amino acids, TMAO, compounds related to energy metabolism (such us intermediates of the TCA), and gut microbial-mammalian co-metabolites such us hippurate. Additionally, they found that alanine was positively associated to blood pressure, while hippurate was negatively associated with it. Most of the metabolites found to be important in driving the difference between East and West groups also appear to be the ones playing the role in the separation of UK-B from UK-C including the increase in %alanine and the decrease in % hippurate. The decrease in %hippurate in the UK-B compared to UK-C could also be related to the absence of alcohol consumption in the UK-B group, since those two variables were negatively correlated in Holmes *et al.* (2008).

A possible reason for the highly significant increase in %DMG in urine of UK-B in comparison to UK-C might may be due to higher homocysteine levels in the blood of UK-B. In fact *N,N*-dimethylglycine is a byproduct of homocysteine metabolism (see Figure 5-15). Homocysteine and betaine are converted to methionine and *N,N*-dimethylglycine by betaine-homocysteine methyltransferase (BHMT) (HMDB00092[15]). DMG is not further metabolised in the liver and the excess is released into the blood and distributed to other body compartments or excreted in urine. It has been proven that Indian Asian men residing in the UK have a higher prevalence of hyperhomocysteinemia than their European counterparts (Chambers *et al.*, 2000). In particular, Bangladeshis living in the East London are known to have higher serum homocysteine levels in respect to the upper

[15] http://www.hmdb.ca/metabolites/HMDB00092

limit of normal for folate-replete people and Caucasians (Obeid *et al.*, 1998). Hyperhomocysteinaemia is associated with an increased risk of cardiovascular disease, especially in non-insulin-dependent diabetics (Hoogeveen *et al.*, 1998).

Furthermore, it has been demonstrated that DMG accumulates in urine and is able to predict elevated plasma homocysteine concentrations (McGregor *et al.*, 2001). Therefore, DMG in urine can be considered to be a proxy of homocysteine blood levels, and the high increase in UK-B compared to UK-C is probably an indication of elevated homocysteine levels in the former group.

Corroborating the idea that the difference in DMG in the UK-B compared to UK-C could be related to homocysteine increase in blood, is the concomitant increase in %betaine as seen in this group. Betaine loss is associated with elevated homocysteine in blood. Betaine is an osmolyte that supplies methyl groups, and controls plasma homocysteine. Abnormal urinary loss of betaine is common in patients with the metabolic syndrome or diabetes mellitus (Lever *et al.*, 2009). Indeed, UK-B volunteers from this study who declared to be affected by diabetes, had higher urinary betaine and DMG than non diabetic volunteers, as reflected in the literature (Dellow *et al*., 1999). However, when eliminating diabetics from the UK-B study group, the significant differences in urinary betaine (p=0.009) and DMG (P=0.001) were still seen.

To further investigate the relationship between arsenic and urinary DMG (as a proxy of homocysteine blood levels), a Spearmann correlation analysis was performed using %DMA, %MMA and %iAs. Both betaine and DMG were significantly positively correlated to %DMA in the urine of the overall UK study group. If one considers that %N-N-dymethylglycine is a proxy of blood homocysteine, the present data shows an indirect positive relationship between homocysteine in blood and %DMA. This is in agreement with the data reported by (Hall *et al.*, 2009) from As-exposed Bangladeshi children, for which blood homocysteine was inversely correlated with %MMA in males (r = −0.27, P = 0.009), and positively to %DMA (r = 0.13, P = 0.10).

Unfortunately, in this study blood samples were not available and blood methionine and homocysteine data are not present for these study groups. However the concomitant and simultaneous increase in DMA, betaine and DMG seen in UK-B compared to UK-C might be linked from the pathway reported by Tseng (Tseng *et al.*, 2009) as shown in Figure 5-15.

According to statistical analysis on the UK-B and UK-C, there was a significant increase in %taurine in urine of UK-B compared to UK-C, identified by both univariate and multivariate approaches, and this represents another interesting point of discussion. Dietary sources of taurine include shellfish, dark meat of turkey and chicken, and cooking does not have an impact on the integrity of the molecule (Wójcik *et al.*, 2010). Both shellfish and chicken were very frequently mentioned in the food diary of the UK Bangladeshis, which may explain their higher urinary taurine levels. Taurine has been suggested to be responsible for lowering blood pressure (Wójcik *et al.*, 2010). The main mechanism through which taurine may decrease blood pressure is thought to be the attenuation of angiotensin II signalling, which causes vasoconstriction and consequently increases BP (Schaffer *et al.*, 2000). Other hypothesese rely on the enhancement of the kinin-kallikrein system in the kidney that causes vasodilation (Kohashi *et al.*, 1983), or on decreased levels of epinephrine, which increases heart rate, and norepinephrine, which causes vasoconstriction. In hypertensive rats supplemented with 1.5% taurine in drinking water for eight weeks, the mean plasma norepinephrine level in the taurine supplemented rats was 383 pg/mL, significantly lower than in the control rats (615 pg/mL) (Yamamoto *et al.*, 1995).

Bophal (Bhopal, 2009) highlights the health paradox regarding Bangladeshis living in the UK. High blood pressure is the dominant risk factor for stroke and should be able to predict stroke mortality. Bangladeshis are known to suffer disproportionally from cardiovascular disease in the UK and the standardized mortality ratio for stroke was 249 (95% CI: 213, 292) for Bangladeshi men according to the 2001 census. Despite the number of deaths from stroke, the UK Bangladeshi population shows a relatively low blood pressure (Bhopal *et al.*, 2005). Several exploratory hypotheses (vitamin D deficiency, infection, combining smoking and chewing tobacco) have been formulated on stroke in Bangladeshis. The adipose tissue compartment overflow hypothesis was presented by Sniderman (Sniderman *et al.*, 2007) to explain the higher risk of diabetes and its precursors, and of cardiovascular disease in South Asians. However, the issue has not yet been solved.

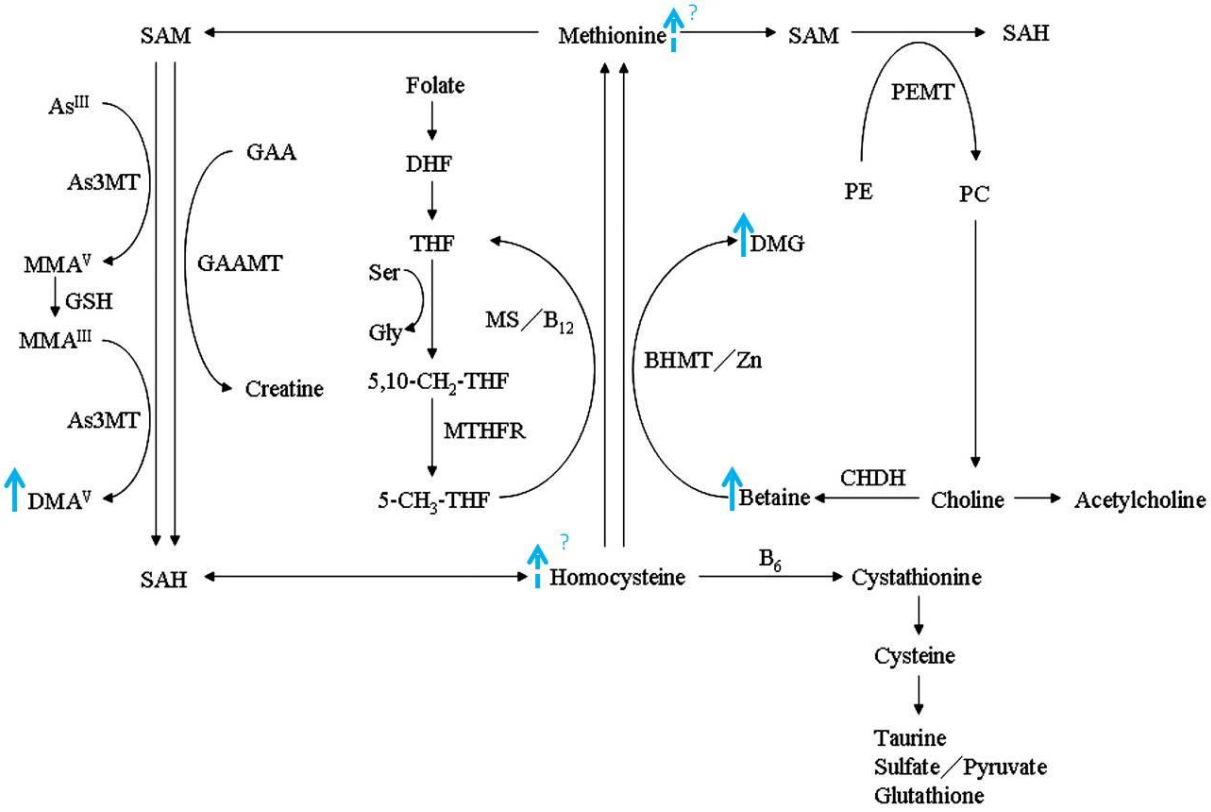


Figure 5-15: Increase in metabolites (upwards pointing blue arrows) of UK-B measured in this study in respect to UK- and the metabolic pathways linking estrogens, choline, betaine, B12, folate and arsenic methylation proposed by Tseng. et al., 2009. Choline and folate both regulate the synthesis of SAM, which is the methyl donor for arsenic methylation. B6, vitamin B6; B12, vitamin B12; BHMT, betaine-homocysteine methyltransferase; CHDH, choline dehydrogenase; DMG, dimethylglycine; GAA, guanidinoacetate; GAAMT, guanidinoacetate methyltransferase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; PC, phosphatidylcholine; PE, phosphatatidylethanolamine; PEMT, phosphatidylethanolamine-N-methyltransferase; SAH, S-adenosylhomocysteine; THF, tetrahydrofolate; 5,10-CH2-THF, methylene tetrahydrofolate; 5-CH3-THF, methyl tetrahydrofolate.

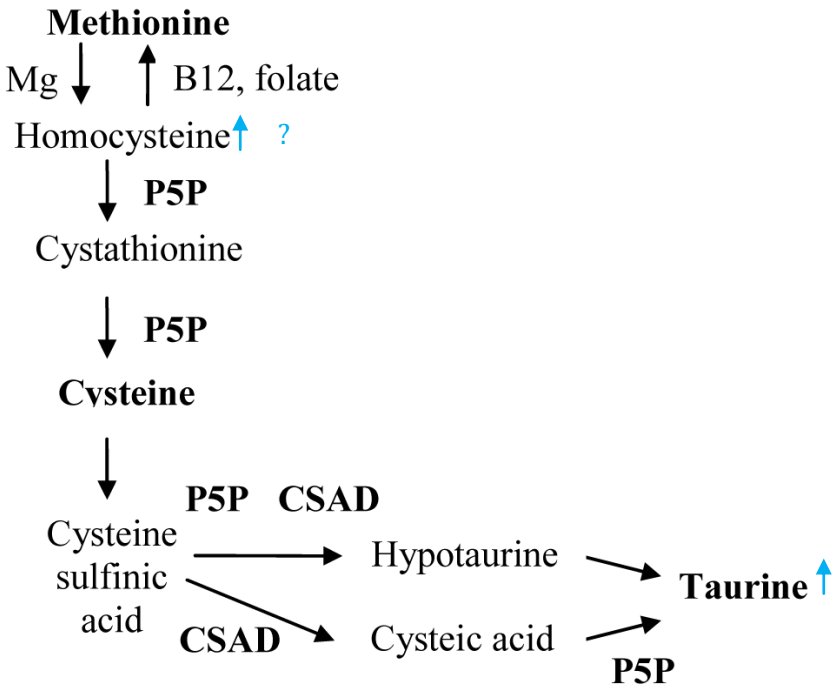


Figure 5-16: Taurine metabolism. Taurine synthesis begins in the liver with a magnesium-catalyzed methylation of methionine to form homocysteine. This process can be reversed by the vitamin B12 and the folate dependent enzyme methionine synthetase. Homocysteine donates its sulfur group to form cystathionine and under the influence of pyridoxal-5'phosphate (P5P) cystathionine is broken down to cysteine. Cysteine, catalyzed by cysteine deoxygenase, combines with dioxygen to become cysteine sulfinic acid, which is then decarboxylated by cysteine sulfinic acid decarboxylase (CSAD) and P5P to hypotaurine. Hypotaurine is oxidized to taurine by hypotaurine dehydrogenase. Alternatively, taurine is formed following the oxidation of cysteine sulfinic acid to cysteic acid and the decarboxylation of cysteic acid by P5P (3). Figure and caption from (Wójcik et al., 2010). Blue arrow indicates the increase in urinary taurine seen in urine of UK Bangladeshi volunteers compared to UK-C.

The screened increase in taurine in UK-B could be the result of either a higher dietary intake of taurine or is a result of a higher taurine production from homocysteine in the UK-B compared to the UK-C. As shown in Figure 5-16, the homocysteine can be converted to cystathione and cysteine and finally leads to the production of taurine. The increased levels of %taurine in urine of the UK-B group compared to the UK-C may be related to low blood pressure and could give a hint to the paradox described by Bhopal (2009).

Other factors possibly impacting on differences in metabolomic profile of UK-B and UK-C were explored. Chewing pan was found to be common amongst the UK-B volunteers. There was no relationship with any of the analysed metabolites, except for %3-

hydroxisovalerate that was found to be significantly decreased in chewers in comparison to non chewers (p=0.028). The reason for this decrease could not be explained.

%Creatine was found to be higher in UK-B females compared to UK-B males. A gender related variation in the content of creatine in the muscles of women has been previously reported (Forsberg *et al.*, 1991) and urinary creatine was found to be higher in the urine of women in comparison to men (Psihogios *et al.*, 2008). Since the groups in the current study were not gender balanced, the decrese in %creatine in the overall UK-B group might be the result of a smaller number of females in this group compared to the UK-C group. Therefore the creatine difference cannot be considered in the inter-ethnicity comparison.

In the UK study group, rice consumption of the UK-B group was demonstrated to be significantly increased compared with the UK-C group (Chaper 3). Rice is a vehicle of arsenic exposure in the UK population. In this chapter, the daily intake of rice was found to be highly correlated to increase in urinary %alanine (p=0.001), %DMG (p<0.001) and %taurine (p=0.020) in the overall UK study group (see Figure 5-17). In contrast, rice intake was negatively correlated to %succinate (p=0.003), %creatine (0.048), %acetate (0.025) and %hippurate (as shown in Figure 5-18). Changes experienced in some of these metabolites might be related to arsenic exposure. If rice is considered as a vehicle of arsenic intake in the UK-B group, the increase in %taurine and the reduction in %hippurate could be the result of arsenic exposure from this route. One study on rats (Wei *et al*., 2009) documents urinary, serum and liver metabolomic changes in response to realgar administration with an increase in urinary taurine and creatine and decrease in urinary levels of trimethylamine-N-oxide, phenylacetylglycine and hippurate, along with signs of impairment of amino acid metabolism. However, it is important to stress that doses of oral exposure in Wei's experiment (Wei *et al*., 2009) were much higher than the dose intake in the current study group.

Figure 5-17 and 5-18 summarise the correlations seen in this chapter by means of a univariate approach between rice intake, urinary DMA and urine metabolites for the UK study group. It is worth noting that there might be partial overlapping of some common effects, including the decrease in %succinate and %hippurate and the increase in %alanine and %DMG. Rice is a demonstrated vehicle of both iAs and DMA, therefore these changes in urine might come from rice intake. Interestingly, %betaine does not correlate to rice intake, but to DMA. It is possible that this increase in betaine is the result of fish

consumption, which also partially contributes to increased urinary %DMA, as explained in Chapter 3.

There are some possible explainations for the increase in %alanine ($P < 0.001$) seen in the UK-B study group compared to the UK-C group:

i. A higher dietary intake of alanine or of a precursor of alanine. Alanine is the 6th most abundant amino acid in rice, while glutamic acid is the most abundant (Houston *et al.*, 1969). Glutamate is converted to alanine and glutamine i*n vivo* in extrahepatic tissues (Said *et al.*, 1989). Moreover rice is positively correlated to %alaine in urine of the UK group. Interestingly, the % alanine in urine of the BD-B group could mean a further contribution from the rice-based diet to this aminoacid intake and excretion.

ii. Data from the literature show that alanine positively correlates with energy intake, dietary cholesterol, body mass index, 24-hour urinary Na and K and inversely with alcohol intake (Holmes *et al.*, 2008). UK-B have no declared alcohol consumers, while the UK-C did. The BMI was higher in UK-B (26.7, SD: 9.9) than in the UK-C (22.1, SD: 8.7). Silman (Silman *et al.*, 1985), in a comparative study of Bangladeshi male immigrants in East London and Caucasians, found a very high fat intake of over 200 g/day (twice the national average). Fat intake accounted for nearly 60% of total energy intake. In addition to the high fat intake, the Bangladeshis showed a higher carbohydrate intake and a higher total energy intake than the national average. Therefore, the increase in alanine could just be the result of these dietary habits and the correlation to rice, as a consequence of the fact that rice, being a staple food of this group, is acting as a proxy of total food intake.

iii. A recent study (Islam *et al.*, 2011) assayed the activity of alanine transaminase (ALT) in serum of arsenic exposed volunteers from Bangladesh. ALT and other serum hepatic enzymes were found to be significantly increased in the high-exposure groups compared to the lowest-exposure groups as signs of liver damage. ALT is normally present in hepatocytes and catalyses according to the following reaction:

Alanine + Oxoglucatic acid = Glutamic Acid + Pyruvic Acid (Equation 7)

In vivo glutamate is converted to alanine and glutamine in extrahepatic tissues, and these compounds can serve as glucose precursors in the liver (Said *et al.*, 1989). If ALT is released in the serum because of hepatic damage, this might cause an accumulation of alanine in blood and then higher excretion in urine. Moreover ALT in blood is able to predict new-onset diabetes independently of classical predictors based on the West of Scotland Coronary Prevention Study (Sattar *et al.*, 2004). The increase in urinary alanine might be the result of an increase in alanine in serum, or of an alteration of the alanine-glucose metabolism in the UK-B. Interestingly, UK-B volunteers affected by dibetes show an increase in %alanine in urine. A significant increase in alanine in urine of patients affected by diabetes has been also reported in the literature (Terentyeva *et al*., 1997).

Eleven diabetic subjects were found in the Bangladeshi study group. Diabetics differed from the non diabetic group for urinary increase in %alanine, %betaine, and %N,N-dymethylglycine. Betaine is known to increase in the urine of patients affected by diabetes. For example, data from Dellow (Dellow *et al.,* 1999) suggest that poor glycemic control is associated with the increase in urinary betaine and retinol binding protein concentrations (a marker of proximal tubular dysfunction) in the urine of diabetic patients. Furthermore, perturbations in the levels of *N,N*-dimethylglycine were found to be indicative of damage to the renal papilla (Gartland *et al.*, 1989, Gartland *et al.*, 1991). Alanine was found altered in the UK-B diabetic group compared to the the UK-B non-diabetic group. Alanine is involved in alanine-glucose metabolism with possible inmplications in diabetes (SPM00127) [16]. Variation in %alanine, %betaine, and %N,N-dymethylglycine are three of the important factors driving the division between UK-B and UK-C. When excluding the diabetic subjects from the analysis, the significant differences between UK-B and UK-C were kept, with an increase in the % of these metabolites in UK-B who did not declare to be affected by diabetes. It would be interesting to understand if this increase is a marker of pre-diabetic status.

16 http://pathman.smpdb.ca/pathways/SMP00127/pathway?reset=true&highlight%5bHMDB00161%5d=true

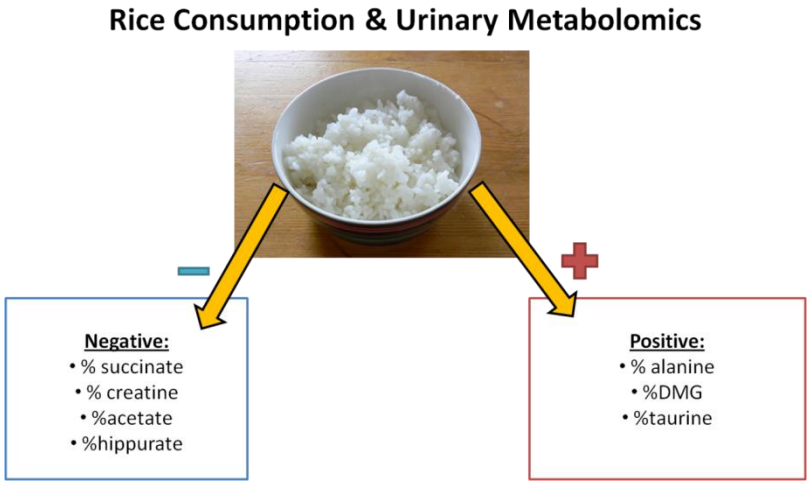


Figure 5-17: Significant correlations seen between daily intake of rice and urinary metabolites in the UK group from this study.

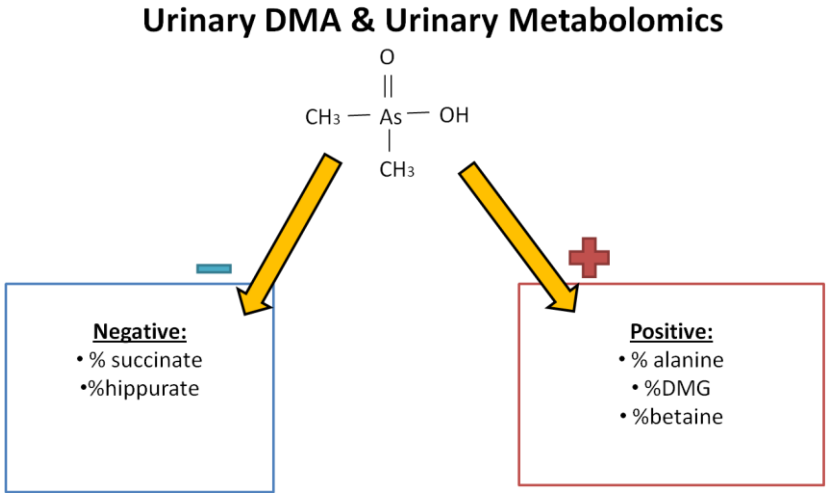


Figure 5-18: significant correlations screened between urinary DMA and urinary metabolites in the UK study group.

When exploring the possibility of correlating total urinary arsenic to metabolomic profile in urine of the UK group, there was generally no correlation with urinary metabolites and it was not possible to fit good quality models. A correlation of total arsenic to %creatinine in UK group and to %dimethylamine in the group from Bangladesh were found. However, when the speciation profile was considered (as % arsenicals in urine), significant relationships started to appear as detailed before. This is not surprising, since total arsenic is in fact not sufficient for evaluating arsenic toxicity because it comes from different dieatary sources. The main lesson from this study is that in order to screen differences related to arsenic in general human populations that are chronically exposed to arsenic *via*

a normal diet, it is vital to consider changes in terms of %DMA, %MA and %iAs. This should be taken into account in future metabolomic studies. When using a multivariate supervised approach (OPLS-DA) to correlate urinary arsenicals to urinary metabolomic profile, models were generally low in predictivity. It is likely that in chronically exposed study groups, other factors such as diet, ethnicity, gender, which normally influence the metabolomic profile, are dominant and are therefore acting to mask the specific effects of arsenic exposure from rice.

For total selenium, it was easier to screen the various relationships. In the UK study group, urinary selenium was positively correlated to %creatinine and %dimethylamine and negatively correlated to betaine. The same trend was seen in the UK Bangladeshi group. Furthermore, in the latter group, other significant features that negatively correlated to urinary selenium were %3-hydroxyisovalerate, %citrate, %glycine, %alanine and %DMG. These trends are complex and cannot be readily explained. However, it is interesting to note the presence of two common features in the UK and BD study groups: The positive correlation of urinary selenium with %creatinine, and the positive correlation with %dimethylamine. Fish consumption is known to significantly increase after exposure to methylamines present in fish. Dimethylamine excretion increases in urine after fish consumption (Zeisel and DaCosta, 1986) and this could be a reason for the increase observed in the current study. In fact, fish is the main source of animal proteins in the BD-B population (Heck *et al.*, 2010) and was also largely consumed in the UK-B group from the current study. Increase of selenium levels are seen after fish consumption in humans (Kresimon *et al.*, 2001). Further investigations are probably needed to understand the relationship between selenium and different urinary metabolites.

UK-Bangladeshis included in this study were all born in Bangladesh and moved to the UK at least from 10 years. Migration is often associated with changes in life-style habits and diet. Even though it is apparent from the food diary that most of the interviewed individuals kept a 'traditional Bangladeshi diet' based on rice and curries, they do tend to eat more food that is also much more varied than Bangladeshis residing in Bangladesh.

In relation to migration, the most striking finding about the BD group was that absolute concentrations of urinary metabolites are affected by country of residence with a large decrease in most of the urinary metabolites in Bangladeshis from Bangladesh in comparison to UK-Bangladeshis, UK-Caucasians and literature values. While urinary metabolites (mM) for UK residents are mainly compatible with those previously reported

in the literature for healthy volunteers (Stella *et al.*, 2006) (Saude *et al.*, 2007), (Shaykhutdinov *et al.*, 2009), urinary metabolomics profiles for BD-B appeared depleted in a number of metabolites and in concentration. Twenty-two metabolites were profiled for BD-B *versus* 29 in the UK resident group; the sum of common profiled metabolites was on average over three times lower in BD-B than in UK residents. The fact that BD-B urine is generally less rich in metabolites is supported by the evidence that SG is significantly lower in BD-B than in UK-B. This could be the result of a poorer diet and the fact the BMI for BD-Bangladeshi was 20.2±3.2 while for UK-B it was 26.7± 9.9. Such a decrease in urinary metabolites could also be the result of higher hydration state in BD-Bangladeshis. Amongst all the metabolites, creatinine, creatine and 3-hydroxyisovalerate show the most pronounced and significant reduction in BD-B compared to UK-B urine. Creatinine is on average about 2.5 times lower than the overall UK group. A high meat diet is known to increase urinary levels of both creatinine and creatine (Xu *et al.,* 2010). Therefore, the reduction in creatinine, creatine and 3-hydroxyisovalerate is probably the result of a lower meat intake and lower BMI in the BD-B group compared to the UK-B group. Dietary investigations on the HEALS population (Heck *et al.*, 2010) revealed that rice is the primary source of protein for the Bangladeshis from Araihazar (BD-B). The second most common source of protein was fish, which accounted for nearly a fifth of the daily protein intake. Milk, eggs, and poultry were not common sources of protein in the HEALS population. If rice contributes to the increase of the %alanine in urine, this could explain the higher % alanine in BD-B compared to UK-B. The BD-B volunteers from the HEALS study are estimated to have a mean protein intake of 67.5 and 78.2 g/day for women and men respectively (Heck *et al.*, 2010). In the HEALS study, a high prevalence of underweight participants was reported. Nonetheless, most participants had adequate protein intake according to the Food and Agriculture Organization standards for body weight (Heck *et al.*, 2010). Indeed, the difference in urinary creatinine and other metabolites found in this chapter seem to better reflect the undernourshiment condition of the BD-B group compared to UK-B than could be accounted for by their apparent daily protein intake. Even if quantitatively sufficient, it is possible that the low meat intake and high plant based food intake in the BD-B results in a lower overall protein intake due to the lower digestibility of plant proteins present in rice and in vegetables. In fact, dietary meat intake is known to improve overall dietary protein quality in terms of digestibility (Millward and Jackson, 2004, Millward, 1999). This variable is higher for meat than for many plant protein sources, especially in the case of diets with whole grains and many legumes that

may contain anti-nutritional factors that adversely influence digestibility (Millward and Jackson, 2004, Millward, 1999). Thus, for vegetable based diets in developed countries, digestibility is likely to be approximately 80% compared with >95% for animal-sourced food and for some plant-food-protein isolates (Millward and Garnett, 2010). In contrast to the B-BD group, UK-B volunteers have a very broad class of foods in their diet, including rice, fish, meat, poultry as indicated by the self-administered 2-day food diary used in this study for the UK group. Therefore, according to this data, creatinine in urine and the general metabolomic profile provides a more pronounced sign of undernourshiment than is apparent from the dietary protein intake estimation. Several human studies have identified associations between indicators of general malnourishment and the development of arsenic-induced skin lesions, skin cancer, and cardiovascular effects (Guha Mazumder *et al.*, 1998, Chen *et al.*, 2003b, Chen *et al.*, 1988, Hsueh *et al.*, 1995). Low intakes of dietary protein can affect arsenic methylation and may increase arsenic-associated toxicity (Steinmaus *et al.*, 2005a). Findings from this study suggest that FFQ might not be *per se* sufficient or sensitive enough to screen for the presence of an adequate intake of amino acid in the population (Heck *et al.*, 2010). Instead, the direct qualitative and quantitative assessement of the urine metabolomic profile might represent a better way to readily detect bioavailable amino acid intake through diet.

The observed decrease in concentration of urinary metabolites in BD-B, should be taken into account for future large scale studies of this population. To overcome the problem, a urine pre-concentration step should be adopted for future NMR analysis of urine from populations living in developing countries with poor nutrition, or more sensitive techniques such as MS sould be used.

Interestingly, for some metabolites (such us threonine, acetate, hippurate), the BD-B show absolute values compatible to the ones for the UK-B group. This results in a larger % contribution than for UK-B. In fact, they are important features driving the separation of the UK-B and BD-B groups. To check for differences in the UK-B and BD-B independently from absolute concentration, which is affected by glomerular filtration rate, the urinary %metabolites of UK-B were compared to those of BD-B.

The two populations (UK-B and BD-B) have different urinary metabolomic profiles as revealed by the OPLS-DA model. Amino acids (%alanine, %threonine, %glycine), %acetate and %hippurate are higher in the BD-B group in comparison with the UK-B group. Conversely %2-hydroxysobutyrate, %3-hydroxyisovalerate, %betaine and %citrate are

lower in comparison to UK-B. The increase in %glycine and %hippurate might be the results of a more vegetarian diet in BD-B than in the UK-B group (Xu *et al.*, 2010, Holmes *et al.*, 2008). These changes in urinary profile associated with migration could be a reflection of the changes in habits, of which diet is amongst the most important changes. The increase of % alanine, %threonine and %glycine could be related to the presence of a more vegetarian diet in BD-B which is mainly based on rice, vegetables and very little of fish (Heck *et al.*, 2010). The UK-B, on the contrary, consume large quantities of non vegetarian foods, including fish, chicken and meat. Vegetarians have higher levels of glycine and threonine in urine than non-vegetarians (Chia and Ton, 2006). Alternatively, as already discussed, the increase in % alanine could be related to higher rice consumption or to increased activity of ALT in blood.

Urinary metabolomics, by means of ^{1}H-NMR, offers a great chance for a rapid and simultaneous detection and quantification of a range of metabolites in urine. This is, to best of the author's knowledge, the first study focusing on the correlation between urinary arsenic and urinary metabolomics in humans. In this study, the effect of migration, ethnicity and dietary changes are possibly playing the main role in driving the differences between UK-C, UK-B and BD-B. Some interesting points have been raised on possible metabolic alterations in relation to arsenic exposure, and these remain to be confirmed with *in vivo* and epidemiological studies.

9. Limitations of this study

One of the limitations of this study is that the populations investigated were not demographically balanced. Therefore differences related to gender, age and other factors might have an impact on the urinary metabolomic profile. Further statistical treatment of the data is in progress in collaboration with Dr Ahsan, Dr Argos and colleagues at Chicago University. Such analysis might change or confirm initial findings resulting from the simple univariate statistics applied in this chapter. For the future, a better stratified sampling strategy should be adopted and a larger population sampled. In fact, a number factors related to inter-individual variability tend to mask the direct effects of arsenic on the urinary metabolomic profile, especially in a general population group exposed to moderate levels of arsenic. Diet is the first confounder in this sense. Future research could be carried out to confirm the apparent correlations with As and Se by carrying out a matched study in Bangladesh residents in areas affected and not affected by arsenic in

ground water. In that way, dietary impact will be minimised and the effect of arsenic effect could be monitored.

10. Conclusions and Future work

In conclusion, this study has applied modern metabolomics approach to explore changes induced by arsenic intake in human metabolomic urinary profile. Metabolomics offer the possibility to readily monitor the effect of a xenobiotic on a biological system. Some possible metabolic alterations are seen in this study in relation to arsenic exposure from rice in a UK-Bangladeshi group. Speciation of urine was vital for this identification of relationships. An increase in %alanine, %N,N-dymethilglycine and % betaine in the UK-Bangladeshis compared to Caucasians, along with the significant positive relationship with %DMA, offer possible points of discussion about the metabolic pathways involved. Rice consumption was related to a positive increase in %alanine and %N,N-dymethilglycine but not in % betaine. Diabetic subjects had higher levels of the cited metabolites in urine, as previously reported. It is remarkable that even subjects considered to be healthy have increased levels of these metabolites in urine with the presence of a distinct metabolomic profile for the UK-B compared to the Caucasians.

A group of Bangladeshi volunteers from Bangladesh, exposed to arsenic from drinking water, were monitored. It was possible to compare the effect of under- and over nourishment in two populations with the same ethnic background. The most pronounced evidence is the reduction of the quality and quantity of metabolites in urine, as well as a decrease in specific gravity. Total urinary arsenic was determined and the only significant positive correlations found were the ones for %dimethylamine. This shows the importance of urinary arsenic speciation in evaluating the effects of arsenic on the metabolomic profile of urine.

Total urinary selenium correlated positively to %dimethylamine and %creatinine in all the monitored groups. In the BD group it shows further correlations, both negative and positive, with other urinary %metabolites.

Diet is playing a great role in masking the effect of arsenic exposure on urinary metabolomic profile in groups that share the same ethnic origin, but have changed their habits as a consequence of migration. The results reveal the complexity of the subject and

provide an interesting starting point for discussion, paving the way for future studies and highlighting the need for awareness about diet and other specific confounding factors.

Finally, the metabolomic study reported here suggests that the overall protein intake in the BD-B is much lower than suggested in the literature. This is probably the consequence of lower digestability of plant-based proteins, which are dominant in BD-B, and highlights the need for public health experts to further monitor the actual protein intake through biomonitoring rather than calculating through food frequency questionnaires.

11. Acknowledgments

Professor Ahsan from the University of Chicago is acknowledged for providing Bangladeshi samples from the HEALS study. Moreover Dr. Argos kindly contributed to statistical treatment of data. An acknowledgement goes to volunteers donating urine samples for this study. I want to thank David Chang from Chenomx for providing support and help in data analysis. A special thank goes to Essam Talha who helped in sample preparation. Thanks to Dr. Griffith of Leicester University for performing the spectroscopic measurements.

Chapter 6

MULTIPLE SCLEROSIS IN THE MT. ETNA REGION: A CASE CONTROL STUDY OF TRACE ELEMENT EXPOSURE

1. Introduction

Mt. Etna (3343 meters, Sicily, Italy) is the highest active volcano in Europe. A prominent feature of Etna's activity is the persistent emission of a volcanic plume arising from summit craters during both quiescent periods and eruptive magma degassing activity (Bagnato *et al.*, 2007) (Figure 6-1). It emits about 16% of the global volcanic heavy metals (including nickel, cadmium, lead) and 19% alkali metals (including sodium, potassium, lithium) during eruptions, and less than 5% in quiet periods (Gauthier and Le Cloarec, 1998). Mt. Etna is an extremely dynamic geochemical system and enrichment in manganese, arsenic, vanadium, selenium and iron is reported in its groundwater as a consequence of a gas-rock-water interaction (Aiuppa *et al.*, 2006; Aiuppa *et al.*, 2003; Roccaro *et al.*, 2007b; Giammanco *et al.*, 1996). These groundwater resources currently represent the only drinking water source for about 1,000,000 people living in the region. Detailed data on the concentrations of trace elements in soil are rare, but initial studies highlight a high concentration of Co (De Vivo, private communication). A small scale food survey characterized the weekly dietary intake for the population of Catania for Ni, Mn, Cr, Pb, Hg and Cd levels (Fallico and Ferrante, 2000; Fallico and Ferrante, 1999). Data are

reported in Table A6-1 of the Appendix to this chapter. Figure 6-2 shows a comparison of estimated nickel dietary weekly intake and Upper Tolerable Intake established for adults by IOM (Food and Nutrition Board, 2001). It is evident from this estimation that the dietary route largely contributes to the weekly intake of Ni.

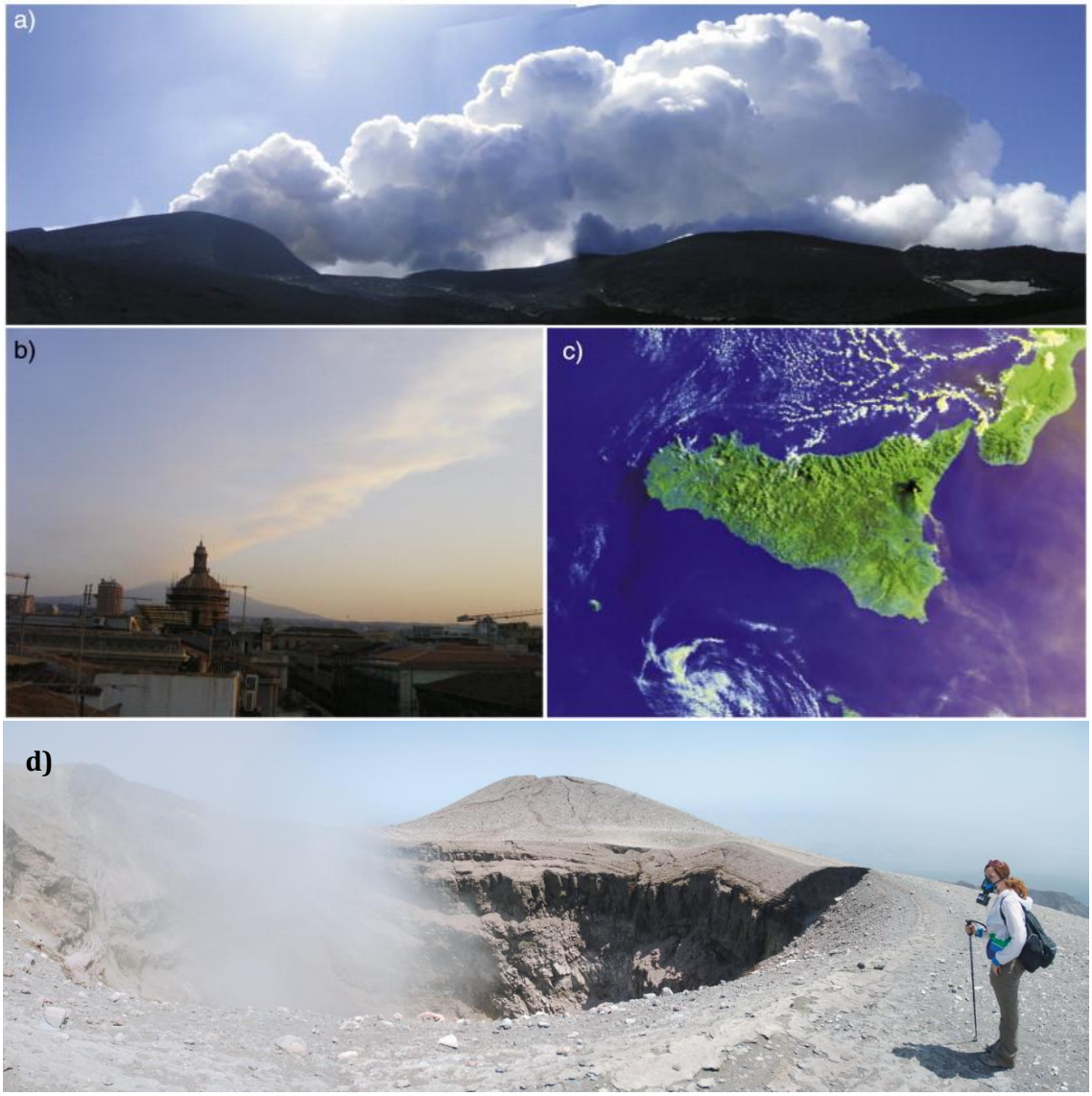


Figure 6-1: Degassing plume observed a) from the summit western slope of the volcano b) from Catania downtown at about at a distance of about 28 km southward from the eruptive vent) (c) from satellite NOAA– (RGB composition) source: (Andronico et al., 2009) d) one of the craters of the Mt. Etna, with evident plume emissions on the left side, Photo by Sergio Calabrese.

Wheat grown in Sicily has a selenium content ranging from 80-120 ppb (Spadoni *et al.*, 2007). Studies on bio-indicators used as a proxy of atmospheric content show the presence of vanadium, chromium, zinc, copper, cadmium and lead in mosses of the Mt. Etna region (Kabata-Pendias and Pendias, 2001; Pais and Jones, 1997). Areas with the

highest concentrations of Ni were found near the southern coast of Sicily, in the vicinity of Palermo and around the Etna Volcano (Gramatica *et al.*, 2006). Studies on sweet chestnut leaves (bio-indicator for plume deposition) collected around Mt. Etna showed that during higher emission rates of gas and ash, leaves accumulate a significant volcanogenic trace metal chemical signal (such as As, Cd, Cu, Mo, Tl, Zn, Rb, Cs and K). As, Cd, Cu, Mo, Tl, K, B, Al and Co showed elevated concentrations downwind of the volcanic source (Eastern flank) in comparison to the other side (Quayle *et al*., 2010).

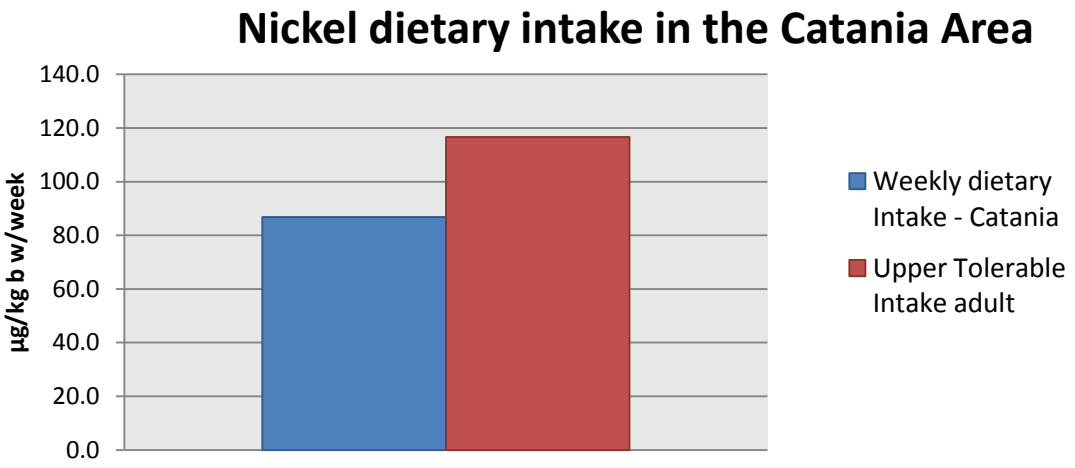


Figure 6-2: weekly dietary intake of nickel as estimated by Fallico et al. (Fallico and Ferrante, 1999). Values represent the average of 4 sampling points in different seasons; Upper Tolerable Intakes[17] were adapted on a weekly basis and considered for someone with 60 kg b.w from (Food and Nutrition Board, 2001).

A possible spatial-temporal cluster of multiple sclerosis (MS) has been reported in the Mt. Etna region in the small town of Linguaglossa (550 mt a.s.l. on the North-East flank of Mt. Etna) (Nicoletti *et al.,* 2005; Nicoletti *et al.,* 2009) (see Figure 6-3). Prevalence rate on January 1st, 2006 was 313.5/100,000 (95% CI 164–462), whereas the average annual onset-adjusted incidence risk was 19.4/100,000 over the last 15 years (1991–2006). Both prevalence and incidence were significantly higher than rates reported in Sicily and in continental Italy, (Nicoletti *et al.*, 2005;, Nicoletti *et al.*, 2001). Incidence risk was significantly higher with respect to the incidence estimated in the same community during

[17] A Tolerable Upper Intake Level (UL) is the highest level of daily nutrient intake that is likely to pose no risk of adverse health effects to almost all individuals in the general population. Unless differently specified specified, the UL represents total intake from food, water, and supplements (Food and Nutrition Board, 2001).

the decade 1981–1990 (3.6/100,000; 95%CI 0.43–83.2) (Nicoletti *et al.,* 2005, Nicoletti *et al.,* 2009).

Figure 6-3: Location of Linguaglossa and some Sicilian cities. Source: Google maps.

Multiple sclerosis (MS) is an autoimmune disorder resulting in inflammatory events, formation of sclerotic plaques and demyelinised lesions within the white matter of the central nervous system. Between 2 and 2.5 million people suffer from MS around the world, of which the greatest proportion are young adults between 20 and 50 years of age. The peak of occurrence is at 30 years of age (Compston *et al.*, 2006c). Women are twice as likely to be affected by MS as men (Compston *et al.*, 2006c). There is a well known irregular geographic distribution of MS (Milo and Kahana, 2010) and ethnic differences in MS frequency; its prevalence varies between < 5/100,000 people in tropical areas and Asia and >100–200/100,000 in temperate areas, especially in large populations of Northern European origin, including United States, Canada, New Zealand and parts of Australia (Compston *et al.*, 2006b; Milo and Kahana, 2010). MS frequency increases with latitude (Kurtzke, 2005), but hotspots are reported in South Mediterranean Europe such as Sardinia (prevalence 157/1,000,000) (Granieri *et al.,* 2000) and Sicily (Savettieri *et al.,* 2001).

"New insights into epistasis and epigenetics have ruled out the possibility of simple causative associations between genes or the environment and MS" (Koch-Henriksen and Sørensen, 2010). In fact, MS is currently considered a multi-factorial disease, and autoimmune reaction causing myelin disruption is probably the interaction between susceptible genes

and loci (i.e. HLA allele DRB1*1501 (Dyment *et al*., 1997) and one or more environmental triggers. Several environmental factors have been hypothesized to be responsible for triggering MS. These include nutrition (Habek *et al*., 2010), vitamin D deficiency (Hanwell and Banwell, 2011), solar radiation (McDowell *et al*., 2010), infectious agents including Epstein–Barr virus (Ascherio and Munger, 2007), *Chlamydia pneumoniae* (Marrie, 2004), the presence of human endogenous retroviruses (Perron and Lang, 2010), smoking (Riise *et al*., 2003) and exposure to chemicals (Schiffer *et al.*, 2001a). However an exhaustive explanation for the causes and mechanism of MS has not yet been found.

An excess or deficiency of trace elements was hypothesised as responsible for the development of MS. For example, a barium hypothesis was proposed (Purdey, 2004) and a deficit of cobalt, manganese, molybdenum and zinc from arable soils in Ukraine was postulated (Zapadniuk, 1992). Additionally, a geographically based MS cluster was reported in the proximity of a zinc smelter in an area with a known excess trace-metal exposure in Illinois (Schiffer *et al.*, 2001a). Foster hypothesizes a connection between iodine and selenium and a range of diseases including MS (Foster, 1993).

A certain number of papers reported the content of trace elements in biofluids of patients affected by MS, with a general lack of common results. For example, Gellein *et al*. (2008) reported a decrease of Mo in the whole blood of patients affected by MS; and Smith *et al*. (1989) reported an altered Cu and Zn homeostasis as a cause or a result from the disease. A large difference for Zn in the whole blood and a marginal difference for copper was found by (Rieder *et al*., 1983) in MS compared to control, while no difference was seen for iron (Fe). Ryan *et al*. (1978) reported a significant difference in the content of some trace elements, including Cu, I, Mn, S, Se, V in scalp hair of MS and controls. Forte *et al.* (2005) found an increase in blood levels of 60 MS for Co, Cu and Ni compared to 60 controls and a decrease of Be, Fe, Hg, Mg, Mo, Pb, Zn. Finally, Ristori *et al*. (2011) recently revealed major contributions of serum Ca, Fe, Sn, Zn, serum antioxidant capacity and serum oxidative status, in discriminating clinically definite multiple sclerosis, clinically isolated syndrome and controls.

Despite the reported excess of MS cases in the volcanic area of Linguaglossa and the proven presence of elevated levels of certain trace elements in the Mt. Etna region, no one has yet attempted an investigation of the possible link between trace element exposure

and MS in this region. MS clusters represent precious opportunities to explore risk factors for MS, including the role of genetics or the environment.

In order to explore the relationship between exposure to essential/non essential trace elements from the volcanic environment and MS, a case-control study was carried out in the city of Linguaglossa. Urine and toenails, well recognised biomarkers of recent and past exposure (Heitland and Köster, 2004; Laohaudomchok *et al.*, 2011), were collected, along with questionnaire data. Total trace element contents in the biological specimens were determined by means of ICP-MS and demographics, dietary and other information collected were used to complement the human tissue analysis.

2. Methods

2.1 Study population

Linguaglossa is located on the North-Eastern side of the Etna volcano. Its official population in 2001, according to the last census data, was 5,422 inhabitants (Istituto Centrale di Statistica, 2001). The population has been fairly constant over the last 20 years and can be considered ethnically stable (Nicoletti *et al.*, 2005). Twelve MS patients out of the 16 MS sufferes previously identified, who experienced the onset of MS during 1990-2006, were enrolled in the study. Samples from the other four patients were unavailable. Background and methods have been extensively reported elsewhere (Nicoletti *et al.*, 2005). Briefly, healthy controls for each enrolled case, matched by age (+/- 5 years), sex and area of residence, were enrolled in the study. Control subjects underwent a standardized neurological examination in order to exclude the presence of neurological disorders.

2.2 Sampling

This study was approved by the Faculty of Health and Life Sciences Human Research Ethics Committee of De Montfort University and by the Ethics Committee of the University of Catania.

During the period February-March 2008, volunteers from the cluster of MS reported in Linguaglossa (Nicoletti *et al.*, 2005), were enrolled as part of a case-control study (Nicoletti *et al*., 2005) along with controls. The Italian Association for Multiple Sclerosis of

Linguaglossa actively contributed to facilitate recruitment and sampling. Twelve MS cases (MS) were enrolled in the study along with 40 matched controls (CNT).

Sampling of urine and toenails was performed by trained specialists. Personal information was collected by the use of an anonymous, self-administered questionnaire. Each volunteer gave a written informed consent.

Each volunteer completed a questionnaire on demographics, dietary habits, body mass index, medications, source of water, occupation, presence of dental fillings, food frequency questionnaire and occupation and kept a 2-day food diary. On the morning of the 3rd day, the volunteers provided mid-stream, first morning urine in 5% nitric acid washed HDPE bottles. Ten toenails (when possible) were collected by the volunteers by the use of stainless steel scissors and stored in the provided sealed plastic bags. Volunteers were not asked to avoid eating fish and seafood in the days before collection, but this information was available thanks to the 2-day food diary.

After collection, urine samples were kept cold till storage at -20°C at the University of Catania (IT). Samples were stored within 3-5 hours from the collection, and during collection, samples were kept at 4°C. The urine samples were sent in dry ice to De Montfort University, Leicester (UK) by express courier. Urine samples were defrosted once to measure specific gravity and to perform a Combur test. Samples were then aliquoted and kept frozen at -20°C till the day of the analysis. Toenails were stored and shipped in sealed plastic bags at room temperature till the day of the analysis.

2.3 Clinical screening and specific gravity

Urine clinical screening was performed with a dipstick (Combur 9, Roche Diagnostics Ltd, UK). Specific gravity (SG) was measured with a digital refractometer (PA/L0S, Atago co. Ltd, Tokyo, Japan).

2.4 Trace elements in urine: preparation and analysis

Urine analysis for trace element composition was carried out at the Health and Safety Laboratory (Buxton, UK). The Health and Safety Laboratory is a United Kingdom Accreditation Service (UKAS) accredited laboratory and successfully participates in the

following Trace Elements External Quality Assessment Schemes: TEQAS[18], GEQUAS[19] and NEQAS[20]. Reference materials used for quality controls in urine included Clincheck level 2 and Biorad level 1 lot 69131.

Nitric acid (Ultrapure) was bought from Romil, UK, and element standards were prepared from multi-elemental standard 100 mg/L (Primar from Fisher, Loughborough UK). Single element ICP-MS standards for internal standards (Rh, Y, In, Pt and Ge) and for Cr, As, Se, Mn and Mo were prepared from 1000 mg/L (BDH, Aristar) ICP-MS standards. EDTA was purchased from Fisher Scientific, Loughborough, UK.

All reagents used were of analytical or higher grade and dilutions were made with high-purity deionised water (>18 MΩ cm).

A Thermo Fisher X series II (Hemel Hempsted, UK) ICP-MS was used for analysis. For some of the analyzed trace elements (Mn, Co, Ni, Cu, Zn, Cd, Tl, Pb), a normal mode (N) was used, while for As, Se and Cr, collision cell mode (CCT) was used in order to contrast polyatomic interferences using 7% hydrogen in He at 3.5ml/min as the collision gas, as shown in Table 6-2.

Table 6-2: ICP-MS Settings and preparation conditions used for urine analysis

Digestion	no
Dilution Factor	1:25
Diluent	For Normal mode: 0.02% EDTA in 1% HNO_3 For CCT: 1% HNO_3
Internal Standard	Rh, Y, In, Ge, Pt
CRM	Clinchek level 2 Biorad level 1
ICP Model	Thermo Fisher X series II
Nebuliser	Burgener Miramist
Mode	CCT for As, Se, Cr N for Mn, Co, Ni, Cu, Zn, Cd, Hg, Tl, Pb
Collision gas	7% H_2 in He (3.5 ml/min)
Analysed isotopes	^{55}Mn, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{68}Zn, ^{111}Cd, ^{205}Tl, ^{208}Pb, ^{52}Cr, ^{75}As, ^{78}Se

[18] http://www3.surrey.ac.uk/SBMS/eqas/teqas.html,

[19] http://www.g-equas.de/

[20] http://www.ukneqas.org.uk/content/Pageserver.asp

For elements analysed in normal mode, calibration standards (0.2-20 µg/L) were used. Urine was diluted 1 in 25 as follows: 0.1 ml urine plus 0.4 ml water and 2 ml of diluent. The diluent was 0.02% EDTA in 1% HNO_3 and was made in 500 ml consisting of: 0.1 g EDTA, 5 ml HNO_3, 0.5 ml 1 mg/L internal standards (Ge, Y, Rh, In, Pt), 0.5ml 1000 mg/L gold (Au) standard. The use of EDTA helped in keeping transition metals in solution.

For As, Cr and Se, analysed using a collision cell, calibration standards had a concentration of 0.1-10 µg/L. Urine was diluted to 1 in 25 (0.1ml urine +0.4ml water and 2ml diluent). In this case, the diluent was made in 1L consisting of: 10ml HNO_3 and 1 ml of 1 mg/L internal standards (Ge, Y, Rh, In, Pt).

2.5 Trace elements in toenails: preparation and analysis

In order to decontaminate from possible external sources of trace elements, toenails were subjected to a careful washing procedure. After a mechanical cleaning step, toenails were chemically cleaned by the use of a multistep washing procedure according to Bass *et al*. (2001), as shown in Table 6-3, using ultrapure grade reagents.

Table6-3:Multistep washing procedure adopted for decontamination of toenails

Solvent	Times
1% triton X	4
Acetone	1
Water	3
Acetone	2

After drying in an oven at 45°C, the toenails were weighed and digested with nitric acid (HNO_3,69.5%, Romil, ultra pure) (250-1000 µl), from room temperature up to 70°C in a hot block system until digestion was complete. Samples were then cooled and diluted up to 10 ml in ultrapure water (>18 MΩ). Samples were filtered through 45 µm filter (Millipore) before analysis. Reference materials (human hair GBW09101, SRM, Republic of China) and procedural blanks were included in the sample batch and treated following the same procedure.

A Multi-element analysis of diluted digestions was performed by ICP-MS (Thermo-Fisher Scientific X-Series II) at Nottingham University, employing a 'hexapole collision cell' (7 % hydrogen in helium) with kinetic energy discrimination (CCT-KED) to remove polyatomic

interferences. Samples were introduced from an autosampler (Cetac ASX-520 with 4 x 60-place sample racks) through a concentric glass venturi nebuliser (Thermo-Fisher Scientific; 1 mL min-1). Internal standards were introduced to the sample stream via a T-piece and included Sc (100 ng m/L), Rh (20 ng m/L) and Ir (10 ng m/L) in 2% trace analysis grade (TAG) HNO_3. External multi-element calibration standards (Claritas-PPT grade CLMS-2 from Certiprep/Fisher) included Al, As, Ba, Bi, Cd, Co, Cr, Cs, Cu, Fe, Mn, Mo, Ni, Pb, Rb, Se, Sr, U, V, and Zn, all in the range 0 – 100 µg /L (0, 20, 40, 100 µg /L). Variable dwell times and three or five analytical runs per analyte were employed. Sample processing was undertaken using Plasmalab software (version 2.5.4; Thermo-Fisher Scientific) using an average blank and sensitivity value (cps/ppb) calculated from calibration blocks typically run every 60 samples. Internal cross-calibration, generated during sample runs, was used for conversion of 'analogue' signals to equivalent values of integrated counts-per-second, where required.

Tuning the instrument prior to analysis was undertaken in standard mode (evacuated hexapole) and verified using a standard performance checking program using Thermo-Fisher bespoke multi-element 'Tune-A' solution (Li, In and U at 5.0 µg /L). The instrument was then set to CCT-KED mode with the following manual changes:

- Hexapole gas controller set to 3.5 mL min^{-1} 7% H_2 in He
- Focus setting to -6.0 V
- Pole bias to -14.0 V
- Hexapole bias to -18.0 V
- D2 lens set to 30 V less negative than standard mode setting.

A performance check for CCT-KED operation mode was then undertaken (Co, In and U at 5.0 µg /L Tune-A solution). The instrument was then left running for 15 minutes prior to calibration with the internal standard (IS) and a blank solution (2% TAG HNO_3) on the IS and sample lines respectively to condition the cones. Typical operating conditions and isotopes used for the analysis are shown in Table 6-4.

Table 6-4: Typical operating conditions used for ICP-MS analysis

Forward power	1404 W
Nebuliser (carrier gas)	0.82 L min^{-1}
Extraction lens	-129.4 V
Lens 1	-729 V
Lens 2	-46.3 V
Lens 3	-195.3
Focus	-6.0 V
D1	-40.8 V
D2	-122 V
DA	-25.1 V
Hexapole bias	-18.0 V
Pole bias	-14.0
Reaction cell gas	3.5 mL min^{-1} of 7% H_2 in He
Quadrupole dwell times	variable
Internal standard	Sc, In, Rh
CRM	human hair GBW09101
Analysed Isotopes	^{51}V, ^{60}Ni, ^{59}Co, ^{55}Mn, ^{78}Se, ^{75}As, ^{66}Zn, ^{65}Cu, ^{95}Mo, ^{56}Fe, ^{208}Pb, ^{238}U

2.6 Statistics

Statistical analysis was performed in Stata, SimcaP+ (Umetrics, Umea) and SPSS (IBM).

3. Results

3.1 Specific Gravity

Urinary specific gravity passed the normality and equal variance tests and was therefore compared using a t-test, no significant difference was found between cases (1.021±0.009) and controls (1.019±0.007) (P = 0.471).

3.2 Combur test

A Combur test was performed on urine to screen for main clinical parameters. Generally MS and controls showed normal values for the screened clinical parameters, however one of the MS cases (A10) showed leucocytes (ca. 500 leuco/µL), nitrite and haemoglobin (ca. 10 erytrocytes/µL) in urine, and case A07 had ketones (50 mg/dL) in urine. They were both secondary progressive forms of multiple sclerosis. One control had glucose in urine.

3.3 Population description and risk factors for MS

Demographics for the patients affected by MS are shown in Table 6-5 and for the overall study population from Linguaglossa in Table 6-6. Data derived from the questionnaires included age, gender, body mass index (BMI) and percentage of smokers. BMI was relatively high for both cases and controls. A higher percentage of smokers were detected in the MS group compared to controls. However when calculating the *odd ratio*, this difference was not significant (P= 0.400). All the volunteers were residing in Linguaglossa but not all of them were born in Linguaglossa; as shown in Table 6-7, 33.3% of the MS cases indicated that they were born in Linguaglossa in contrast to 55.6% of the controls.

Univariate logistic regression was used to screen the presence of risk factors emerging from questionnaires and the results are shown in Table 6-8. None of the investigated factors appeared to be associated with higher risk of developing MS.

Drinking water and food are possible vectors of trace elements for the general population. A specific section of the questionnaire was dedicated to water intake and source (tap/bottle/private well).

Figure 6-4 reports the answer to the questions on tap and well water (both groundwaters) consumption in the overall population. 42 % of the interviewed volunteers declared the use of tap water as their main drinking water source and 85% drink this source of water to variable extent. Moreover 100% of the people interviewed indicated using tap water for cooking purposes. This data indicated that, in general, the local population is largely dependent on the use of the Etnean aquifer for both drinking and cooking purposes. Finally 87% of the interviewed subjects declared to eat locally grown food to some extent.

Table 6-5: MS cluster description

ID	MS type[1]	GENDER	AGE	BMI
A01	RR	F	38	24
A02	RR	M	43	33
A05	RR	F	27	22
A06	SP	F	59	29
A07	SP	F	42	23
A08	RR	F	29	25
A10	SP	M	65	23
A100	RR	F	38	28
A101	RR	F	23	24
A11	RR	F	23	18
A12	SP	F	47	15
A15	RR	F	41	21

([1]) Relapsing remitting (RR), secondary progressive (SP)

Table 6-6: Demographics and basic information on the study population

	Cases (n=12)	Controls (n=40)
Age	44 ± 12	40 ± 13
% males	17%	34%
BMI	23.8 ± 4.9	25.9 ± 4.2
% smokers	42%	32%*

(*) percentage calculated on the number of volunteers that answered a question on smoking habit.

Table 6-7: Place of birth

Place of Birth	Controls	Cases	Total
ABROAD	7.4% (2)	8.3%(1)	7.7%(3)
MAINLAND ITALY	3.7% (1)	0% (0)	2.6% (1)
CATANIA (CITY)	7.4% (2)	33.3% (4)	15.4% (6)
FIUMEFREDDO*	3.7% (1)	8.3% (1)	5.1% (2)
LINGUAGLOSSA	55.6% (15)	33.3% (4)	48.7% (19)
MALETTO*	0% (0)	8.3% (1)	2.6% (1)
PIEDIMONTE*	18.5% (5)	8.3% (1)	15.4% (6)
MESSINA	3.7% (1)	0% (0)	2.6% (1)
Total	100% (27)	100% (12)	100% (39)

Numbers in brackets represent the actual number of counts, percentage is calculated on the total number of volunteers that answered this question among cases and controls separately. *Towns in the Mt. Etna region in the proximity of Linguaglossa

Table 6-8: Factors investigated by mean of univariate logistic regression, odds ratios (OR), confidence intervals (CI) are reported in brackets

Factors	OR (CI)	P
Habits		
Smoker	1.49 (0.38-5.78)	0.561
Supplements	3.5 (0.61-20.72)	0.158
Sport	1.4 (0.35-5.60)	0.612
BMI>50th	0.38 (0.086-1.66)	0.197
Drinking		
Bottle water[1]	1.1 (0.25-4.82)	0.889
Tap water[2]	2.26 (0.25-20.50)	0.467
Well water[3]	1.94 (0.51-7.44)	0.329
Past bottle[4]	3.4 (0.19-59.37)	0.402
Drink 1 L water/day	0.55 (0.13-2.34)	0.417
Drink 2 L water/day	0.259 (0.026-2.529)	0.245
Coffee ≤50th (14 cups/week)	0.329 (0.068-1.603)	0.169
Coffee >50th (14 cups/week)	0.23 (0.0350-1.539)	0.130
Tea>50th	2.1 (0.434-10.168)	0.357
Soft drink>50th	1.093 (0.244-4.890)	0.907
Juice>50th	3.3 (0.701-15.86)	0.130
Alcohol	1.27 (0.328-4.889)	0.732
Diet		
Organic Beef	0.875 (0.154-4.972)	0.880
Organic Chicken	0.805 (0.144-4.501)	0.805
Organic Fruit	0.480 (0.089-2.60)	0.395
Organic Vegetables	0.341 (0.064-1.840)	0.212
Locally grown Vegetables	0.800(0.286-2.237)	0.671
Sea fish in the last week	1.556 (0.362-6.681)	0.552
Chicken in the last week	1.33 (0.347-5.061)	0.679
Mushrooms in the last week	1.32 (0.35-5.06)	0.679
Others		
Dye hair	1.45 (0.37-5.68)	0.590

(1) Current use of bottle water as drinking source, (2) current use of tap water as drinking source, (3) current use of well water as drinking source (4)use of bottle water in the past.

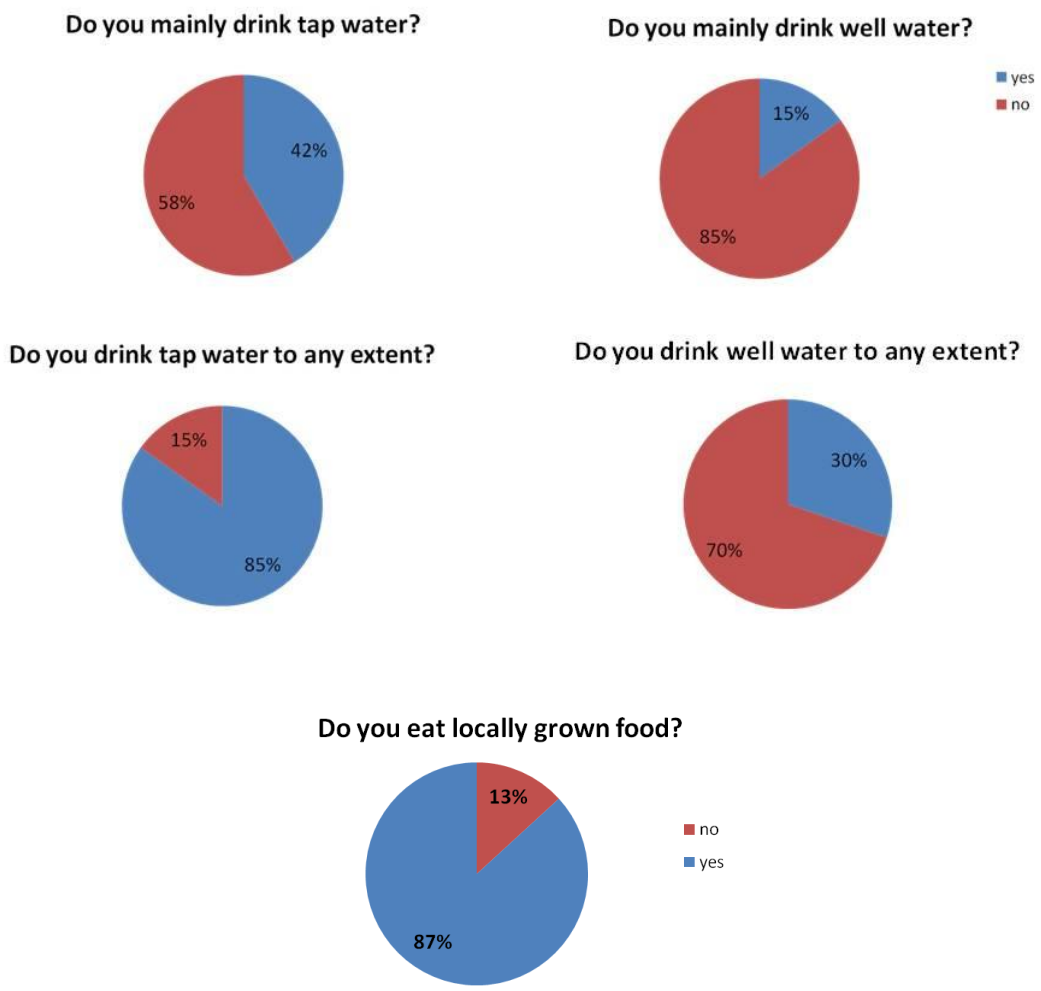


Figure 6-4: Groundwater and locally grown food consumption in the city of Linguaglossa.

3.4 Quality checks: urine

The Limit of Detection (LOD) was calculated as three times the standard deviation of the procedural blanks and multiplied by the dilution factor according to the European standard prEN 13804 as shown in Table 6-9.

For those cases with values < LOD, they have been substituted with LOD/2 in order to perform statistical analysis. Certified and measured values for urine reference materials and % recovery are shown in tables for Biorad level 1 CRM (Table 6-10 A and B) and Clincheck level 2 CRM (Table 6-11 A and B), respectively. All the elements reported % recovery between 71 and 132% for Biorad level one and between 86 and 106% for clincheck level 2.

Table 6-9: LODs for elements analysed in normal mode in urine, values are expressed in µg/L

	Mn	Co	Ni	Cu	Zn
LOD	1.032	0.037	0.226	1.81	21.8
	Cd	**Tl**	**Pb**	**Cr**	**As**	**Se**
LOD	0.040	0.0006	0.079	0.202	0.998	0.540

Table 6-10A: Biorad level I – Elements measured in normal mode [µg/L]

Element	Mn	Co	Ni	Cu	Zn	Cd	Tl	Pb
QC Range	6.03-9.05	5.45-8.17	2.34-6.67	8.08-12.1	305-457	7.22-10.8	6.83-10.3	11-16.5
Certif.	7.5	6.8	4.5	10.1	381	9.01	8.565	13.75
1	7.0	6.5	3.2	7.9	329	8.8	8.3	13.5
2	7.0	6.4	3.1	7.8	319	8.6	7.9	12.9
3	8.0	6.6	3.4	8.7	351	8.7	8.0	13.0
4	8.6	6.4	3.3	8.5	331	8.4	7.9	12.7
5	8.2	6.3	3	8.6	327	8.5	7.8	12.9
6	8.1	6.3	3.3	8.4	323	8.4	8.0	13.0
7	8.3	6.5	3.2	9.0	321	8.8	8.3	13.7
8	8.0	6.4	3.1	8.8	337	8.7	8.3	13.8
9	8.0	6.3	3.0	8.5	324	8.5	8.3	13.5
% Rec	**105**	**94**	**71**	**84**	**86**	**95**	**94**	**96**

Table 6-10B: Biorad level I – Elements measured in CCT mode [µg/L]

Element	Cr	As	Se
QC range in µg/L	0.758-1.67	52.1-78.1	57.8-86.2
Certified	1.21	88.5	72.0
1	1.43	84.0	62.2
2	1.81	80.5	59.5
3	1.70	79.7	60.7
4	1.65	79.5	58.7
5	1.45	80.9	60.6
6	1.56	78.6	60.5
7	1.85	79.2	57.8
8	1.52	79.1	57.9
9	1.60	75.3	58.3
10	1.40	81.9	62.7
% rec	**132**	**90**	**83**

QC range indicates the certified range and Certif represents the certified value

Table 6-11A: Clinchek level II - Elements measured in normal mode [µg/L]

Element	**Mn**	**Co**	**Ni**	**Cu**	**Zn**	**Tl**	**Pb**
QC Range	17-25.4	27.7-41.5	33.1-55.1	94.4-142	445-667	15.0-22.6	48.4-80.6
Certified	21.2	34.6	44.1	118	556	18.8	64.5
1	18.2	31.3	40.5	107	518	17.1	61.8
2	18.4	31.4	40.5	108	504	16.8	60.4
3	19.9	32.1	42.9	113	511	16	57.6
4	20.8	32.2	42.8	114	529	16.1	58.3
5	19.4	30.8	40.7	120	510	15.7	56.5
6	19.6	31.4	41.7	111	514	15.7	57.5
7	19.6	31.2	41.7	112	511	15.9	57.6
8	19.5	31	40.9	111	514	16	57.8
9	19.6	31.3	41.2	111	519	15.7	56.6
% Rec	**92**	**91**	**94**	**93**	**93**	**86**	**90**

Table 6-11B: Clinchek level II - Elements measured in CCT [µg/L]

Metal	**Cr**	**As**	**Se**
QC range in µg/L	28.3-42.5	65.7-98.5	61.2-91.8
Certified	35.4	83.9	76.5
1	37.8	86.8	66.2
2	39.3	84.4	69.5
3	38.3	85.8	67.7
4	39.1	86.1	65.6
5	38.1	82.5	65.6
6	36.6	80.4	63.5
7	37.1	83.4	61.5
8	37	79.1	63.8
9	36.8	82.6	63.7
10	35.9	79.6	62.9
% Rec	**106**	**99**	**85**

QC range indicates the certified range and Certif represents the certified value

3.5 Quality check: toenails

Table 6-12 reports LOD, LOQ and recoveries for toenail analysis. LOD was calculated as 3 *DS of the procedural blanks and LOQ was calculated as 10*SD procedural blanks divided by average sample weight, which was 57 mg. Recoveries for Mn, Fe, Cu, Z, As, Sr and Ba varied from 77-118%, as shown in Table 6-12. V, Ni and Se in hair reference material were lower than 50%, therefore for these elements, the data was considered in a semi-

quantitative manner and is limited for purposes of comparison within the Linguaglossa group.

Table 6-12 Quality checks for trace elements in toenails

Element	V	Mn	Fe	Co	Ni	Cu
CRM-1[2]	0.038	2.208	50.8	0.09	1.49	21
CRM- 2[2]	0.032	2.097	62.9	0.07	1.55	19
Certified value[1]	(0.069)	2.94± 0.20	71.2 ± 6.6	0.135 ± 0.08	3.71 ±0.40	23 ± 1.4
Recovery	**48%**	**77%**	**118%**	**63%**	**45%**	**82%**
LOD [1]	0.013	0.481	31.2	0.041	0.381	8.6
LOQ [2]	0.001	0.028	1.8	0.002	0.022	0.5
Element	**Zn**	**As**	**Se**	**Sr**	**Ba**	**U**
CRM-1[2]	145	0.53	0.38	3.869	5.08	0.0033
CRM- 2[2]	150	0.52	0.33	4.059	5.13	0.0033
Certified value[1]	189 ± 8	0.59 ±0.07	0.58±0.05	4.19±0.14	(5.41)	/
Recovery	**77%**	**85%**	**48%**	**92%**	**93 %**	**NC**
LOD[3]	13.2	0.024	0.235	0.080	0.276	0.002
LOQ [2]	0.8	0.001	0.014	0.005	0.016	0.0001

(1) Indicated as mean ± standard deviation, values in brackets are not certified, (2) [µg/g]; (3) [µg/l]

3.6 Trace elements in urine: MS do not differ from CNT

Urinary concentrations for the eleven trace elements analysed were not normally distributed; a total of 49 urine samples were analysed for trace element analysis and the full data set is reported in tables A6-2 and A6-3 of the Appendix. Descriptive statistics for the two study groups are reported in before SG correction (in Table 6-12A) and after SG correction using the formula reported by (Nermell *et al.*, 2008c) (in Table 6-12B) :

U-As SG [µg/L] = (Urinary Arsenic [µg/L]) X (1.019-1/(Specific Gravity - 1.000)) (Equation 8)

Box plots in Figure 6-5 show urinary concentration (before SG correction) for selected elements in MS and CNT. No significant difference (Mann-Whitney Rank sum) could be appreciated between the cases and controls before and after SG correction, except for chromium, which was higher in the urine of MS patients, but only after SG correction. The absence of a particular urinary profile for this MS group is also confirmed by a principal component analysis (PCA) (see Figure 6-6). This shows that MS (blue squares) and CNT (red triangles) did not cluster separately.

Table 6-13A: Descriptive statistics for urine of cases of multiple sclerosis (MS) and controls (CNT) [µg/L] without correction.

n=49	Mn	Co	Ni	Cu	Zn	Cd	Tl	Pb	Cr	As	Se
Geomean	1.7	0.4	3.2	12.6	265	0.37	0.25	0.992	0.74	31.5	28.4
stdev	0.8	0.7	2.4	8.1	367	0.35	0.62	2.13	0.30	108	18.0
min	< LOD	0.1	0.6	3.4	< LOD	0.07	0.05	< LOD	0.19	11.5	10.2
max	4.7	3.6	11.0	39.7	1855	1.47	4.27	10.5	1.98	773	79.7
60th	2.1	0.5	3.5	14.1	351	0.43	0.27	1.17	0.85	35.0	37.3
95th	2.8	1.9	8.4	30.3	839	1.27	0.81	5.70	1.20	77.8	62.7
Mean	1.9	0.6	3.9	14.5	381	0.49	0.39	1.64	0.80	49.7	33.2
Median	1.9	0.4	3.2	12.5	285	0.39	0.24	0.954	0.79	30.1	30.1
MS=12	Mn	Co	Ni	Cu	Zn	Cd	Tl	Pb	Cr	As	Se
Geomean	1.9	0.4	3.0	13.8	172	0.35	0.25	0.821	0.59	36.8	29.1
stdev	0.5	0.5	2.0	8.1	251	0.40	0.38	2.60	0.35	33.5	20.5
min	1.3	0.05	1.0	5.4	< LOD	0.07	0.08	0.103	0.19	12.8	11.4
max	2.7	1.8	7.0	29.6	906	1.28	1.36	9.69	1.27	136	70.9
60th	2.3	0.7	3.3	17.4	283	0.50	0.28	0.97	0.70	42.1	39.8
95th	2.6	1.6	6.6	27.3	728	1.09	1.07	5.15	1.18	98.1	65.6
Mean	2.0	0.6	3.5	15.9	292	0.51	0.36	1.54	0.68	45.5	34.6
Median	2.1	0.6	3.0	15.9	258	0.37	0.23	0.891	0.53	39.1	25.5
CNT=37	Mn	Co	Ni	Cu	Zn	Cd	Tl	Pb	Cr	As	Se
Geomean	1.7	0.4	3.3	12.2	298	0.4	0.3	1.056	0.80	30.0	28.2
stdev	0.8	0.7	2.5	8.1	396	0.3	0.7	1.996	0.27	123	17.4
min	<LOD	0.1	0.6	3.4	41	0.1	0.0	0.040	0.43	11.5	10.2
max	4.7	3.6	11.0	39.7	1855	1.5	4.3	10.5	1.98	773	79.7
60th	2.0	0.4	3.8	12.9	364	0.4	0.3	1.25	0.86	32.1	36.7
95th	2.8	2.0	8.8	32.2	960	1.3	0.8	5.04	1.16	66.4	58.5
Mean	1.9	0.7	4.0	14.1	409	0.5	0.4	1.67	0.84	51.0	32.7
Median	1.9	0.4	3.2	12.4	298	0.4	0.2	0.96	0.81	27.8	33.6
	Mn	Co	Ni	Cu	Zn	Cd	Tl	Pb	Cr	As	Se
P (Mann-Withney)	0.538	0.523	0.500	0.500	0.317	0.889	0.889	0.329	0.122	0.193	0.789

Table 6-13B: Descriptive statistics for urine of cases of multiple sclerosis (MS) and controls (CNT) [µg/L] after SG correction.

n=49	Mn	Co	Ni	Cu	Zn	Cd	Tl	Pb	Cr	As	Se
Geomean	1.8	0.47	3.5	13.7	287	0.40	0.28	1.13	0.80	34.2	30.8
stdev	1.3	0.66	2.2	8.3	283	0.37	0.51	1.92	0.46	100	17.2
min	<LOD	0.03	0.3	1.4	<LOD	0.05	0.02	0.06	0.11	1.9	2.9
max	6.2	3.62	10.7	46.2	1400	2.00	3.25	8.02	3.14	670	86.4
60th	2.1	0.56	4.1	16.7	354	0.58	0.30	1.34	0.94	39.7	36.5
95th	4.8	1.84	8.5	29.8	895	1.16	1.19	6.82	1.38	114	64.6
Mean	2.2	0.68	4.1	15.8	381	0.52	0.41	1.74	0.90	54.1	35.6
Median	1.8	0.48	3.7	14.7	269	0.47	0.26	1.12	0.85	30.0	34.3
MS=12	Mn	Co	Ni	Cu	Zn	Cd	Tl	Pb	Cr	As	Se
Geomean	2.2	0.51	3.3	15.5	207	0.39	0.28	0.9	0.7	41.1	32.6
stdev	1.6	0.29	0.8	4.9	169	0.23	0.22	1.7	0.3	48.9	7.1
min	1.0	0.16	2.3	7.9	<LOD	0.10	0.13	0.2	0.3	15.9	19.7
max	5.3	1.16	4.8	25.6	573	0.81	0.86	6.6	1.2	184.3	46.4
60th	2.0	0.53	3.8	17.6	246	0.57	0.27	1.1	0.7	41.7	35.9
95th	5.0	1.09	4.5	23.5	533	0.73	0.70	3.8	1.1	137.0	42.2
Mean	2.6	0.57	3.4	16.2	280	0.46	0.33	1.4	0.7	54.3	33.3
Median	1.9	0.48	3.4	16.5	232	0.55	0.25	0.9	0.7	33.8	35.2
CNT=37	Mn	Co	Ni	Cu	Zn	Cd	Tl	Pb	Cr	As	Se
Geomean	1.7	0.5	3.6	13.1	330	0.41	0.28	1.2	0.9	32.2	30.2
stdev	1.2	0.8	2.4	9.2	306	0.41	0.58	2.0	0.5	113	19.4
min	0.3	0.0	0.3	1.4	85	0.05	0.02	0.1	0.1	1.9	2.9
max	6.2	3.6	10.7	46.2	1400	2.00	3.25	8.0	3.1	700	86.4
60th	2.1	0.6	4.5	16.3	361	0.58	0.31	1.4	1.0	34.3	38.3
95th	3.7	2.0	8.9	32.9	1030	1.26	1.34	7.0	1.4	78.9	67.8
Mean	2.1	0.7	4.3	15.6	414	0.54	0.44	1.9	1.0	54.0	36.3
Median	1.8	0.5	3.8	14.1	292	0.43	0.28	1.2	0.9	30.0	33.8
	Mn	Co	Ni	Cu	Zn	Cd	Tl	Pb	Cr	As	Se
P (Mann-Withney)	0.659	0.515	0.340	0.317	0.209	0.907	0.963	0.245	**0.039**	0. 500	0.889

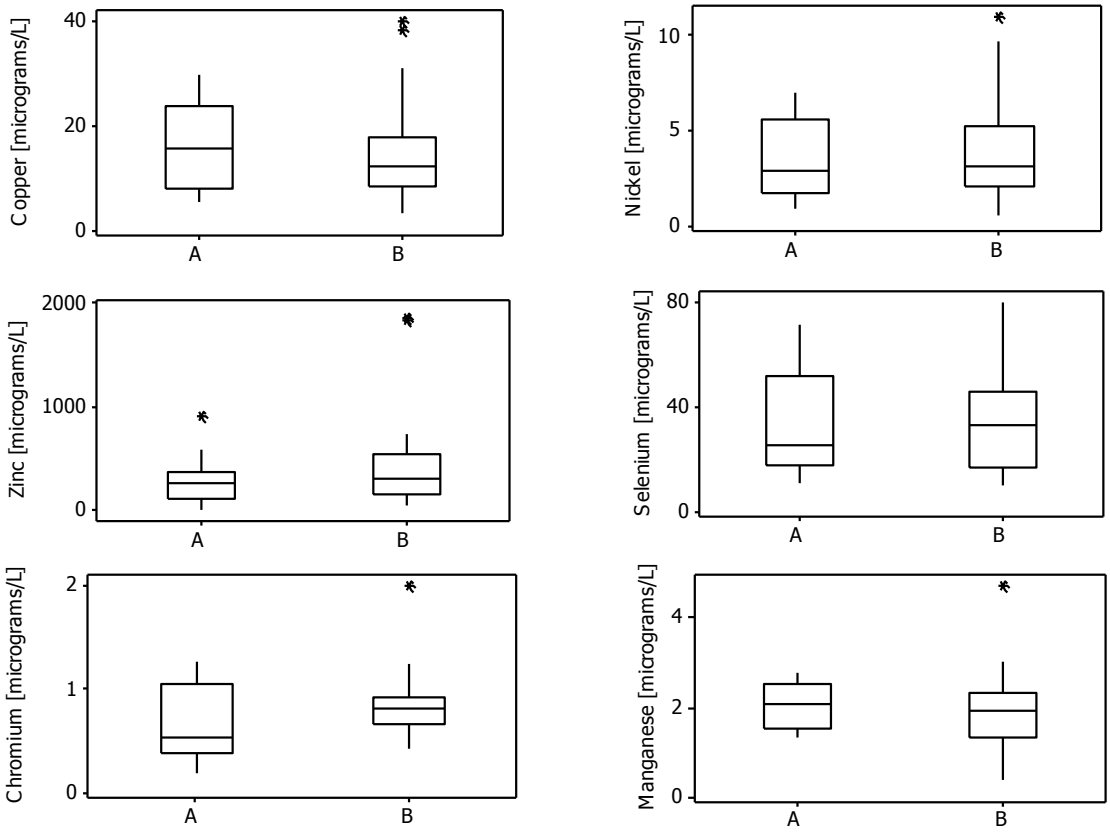
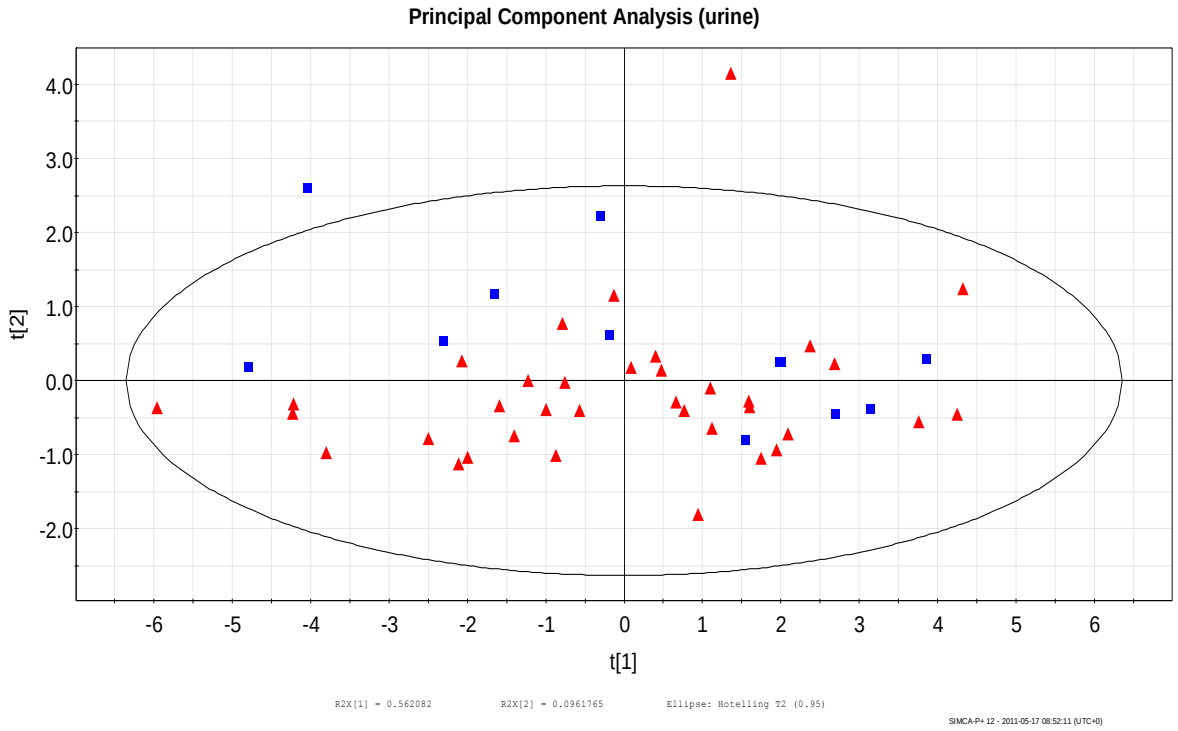


Figure 6-5: Box plots for some of the studied trace elements in urine samples of the Linguaglossa study population. Controls (CNT, n=37) and multiple sclerosis cases (MS, n=11).



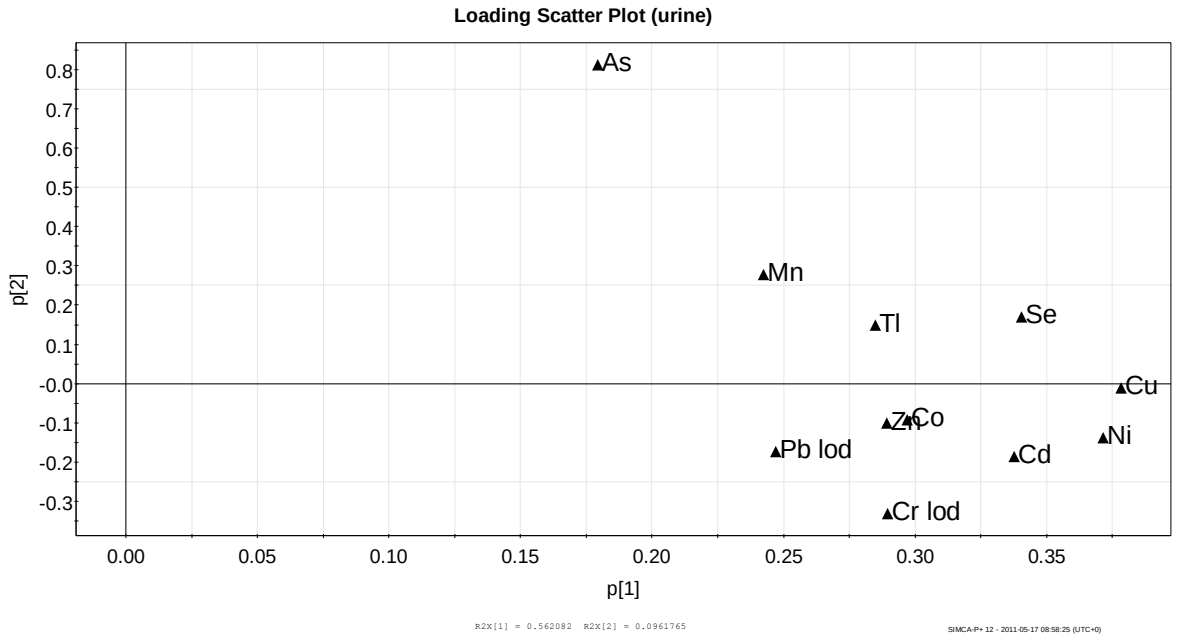


Figure 6-6: a) PCA of trace elemental urinary composition for the Linguaglossa study group, blue squares represent MS and red triangles represent CNT; b) loading scatter plot of the variables.

3.7 Trace elements in toenails

A total of forty-seven (10 MS and 37 controls) toenails samples were analysed because some of the volunteers did not provide a sufficient quantity of toenail samples to carry out the analysis. Descriptive statistics for toenail concentrations and the results of a Mann Whitney test are shown in Tables 6-14A and 6-14B, while the full dataset is reported in Table A6-4 of the Appendix.

There was no significant difference between the MS and CNT for the majority of the trace elements investigated, however a decrease in toenail concentrations (see Figure 6-7) for V, Ni, Co, Mn, Zn, Cu, Fe and U in MS compared to CNT was evident. Conversely, Mo was higher in MS than CNT. As was quite similar in the toenails of MS and CNT.

Table 6-14A: Descriptive statistics for trace elements in toenails [µg/g]

[µg/g]	V	Mn	Fe	Co	Ni	Cu	Zn
GEOMEAN	0.029	0.40	14.9	0.007	0.14	2.92	93
ST DEV	0.040	0.97	12.8	0.015	0.38	1.23	20
MIN	0.004	0.06	0.9	0.001	0.01	0.97	59
MAX	0.233	6.24	64.4	0.096	2.24	8.88	166
60th	0.036	0.43	18.4	0.012	0.18	3.11	95
90th	0.078	1.08	36.3	0.022	0.56	4.09	118
MEAN	0.040	0.64	18.6	0.012	0.27	3.11	95
MEDIAN	0.033	0.35	14.9	0.009	0.15	2.96	91
MS (n=10)	**V**	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**
GEOMEAN	0.021	0.41	10.4	0.008	0.09	2.47	84
MEDIAN	0.029	0.38	14.5	0.009	0.10	3.01	87
60th	0.035	0.39	16.2	0.012	0.12	3.07	92
90th	0.041	1.61	22.3	0.024	0.27	3.29	98
CNT (n=37)	**V**	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**
GEOMEAN	0.032	0.40	16.5	0.007	0.16	3.05	95
MEDIAN	0.034	0.34	14.9	0.009	0.15	2.89	93
60th	0.036	0.46	19.2	0.012	0.21	3.15	95
90th	0.100	1.06	39.0	0.022	0.71	4.16	119
	V	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**
P (Mann-Withney)	0.292	0.928	0.317	0.866	*0.081*	0.384	0.217

Table 6-14B: Descriptive statistics for trace elements in toenails [µg/g]

[µg/g]	As	Se	Sr	U	Ba
GEOMEAN	0.091	0.654	0.90	0.012	0.42
ST DEV	0.607	0.293	0.88	0.010	1.02
MIN	0.029	0.204	0.01	0.002	0.02
MAX	4.071	2.072	3.89	0.058	6.72
60th	0.086	0.775	1.14	0.014	0.50
90th	0.202	0.932	2.27	0.023	1.14
MEAN	0.20	0.71	1.22	0.01	0.69
MEDIAN	0.08	0.72	1.02	0.01	0.45
MS (n=10)	**As**	**Se**	**Sr**	**U**	**Ba**
GEOMEAN	0.105	0.597	0.58	0.008	0.31
MEDIAN	0.058	0.705	0.83	0.009	0.31
60th	0.078	0.772	1.03	0.009	0.48
90th	0.512	0.937	1.89	0.022	1.61
CNT (n=37)	**As**	**Se**	**Sr**	**U**	**Ba**
GEOMEAN	0.088	0.671	1.02	0.013	0.46
MEDIAN	0.075	0.719	1.05	0.014	0.46
60th	0.086	0.781	1.14	0.015	0.50
90th	0.176	0.932	2.27	0.023	1.03
	As	**Se**	**Sr**	**U**	**Ba**
P (Mann-Whitney)	0.668	0.745	0.507	***0.028***	0.443

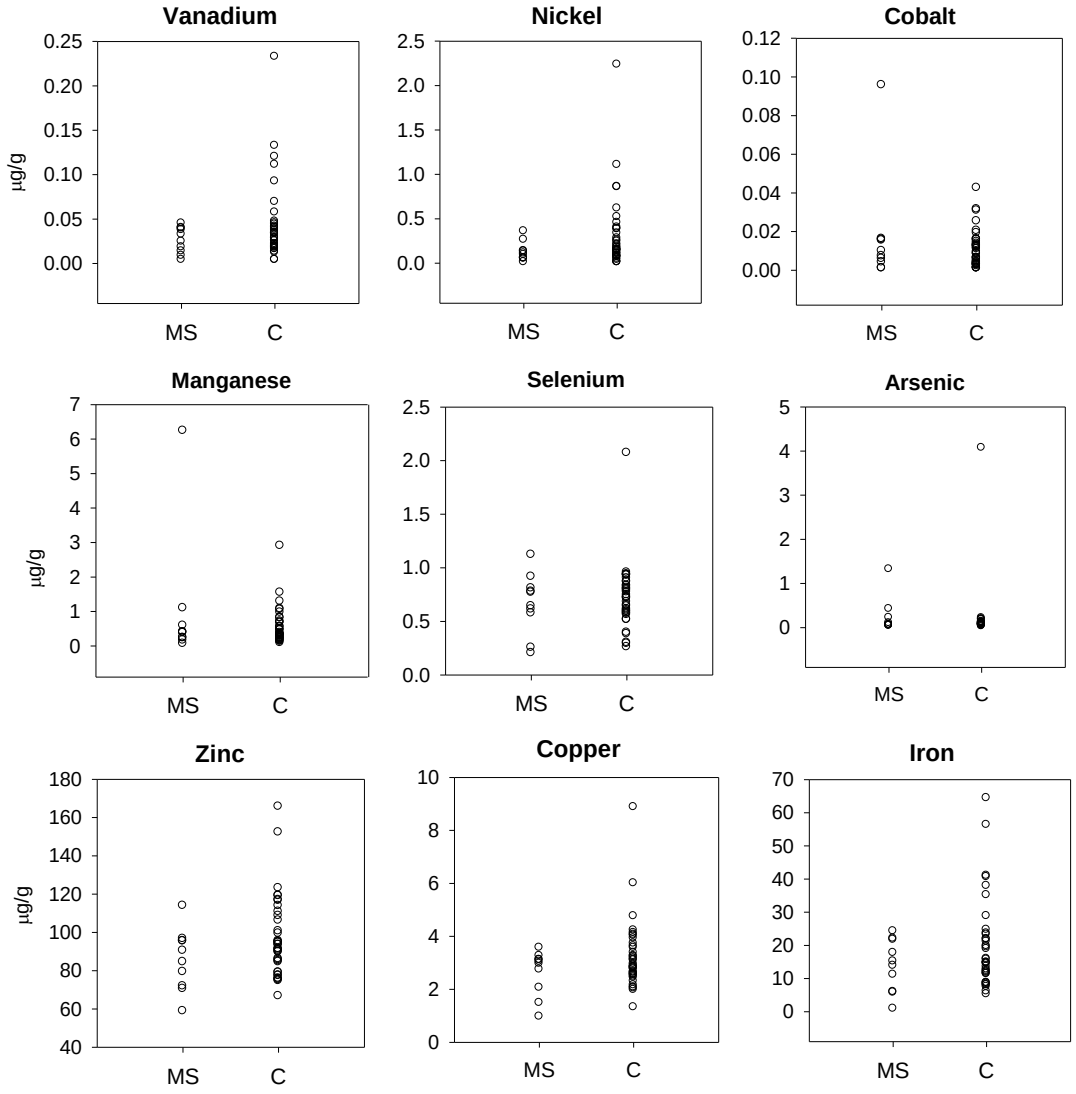


Figure 6-7: Trace elements in toenails of volunteers from the Mt. Etna region: multiple sclerosis patients (MS) and controls (C) are represented as dots.

3.8 Comparing urine trace element profile to literature: assessing the 'Mt. Etna effect'

In order to assess the environmental exposure to trace elements in the overall Linguaglossa population, a comparison to literature reported values was performed as shown in Table 6-15. This table includes non-occupationally exposed populations from Germany (Heitland and Köster, 2006), from the UK (values reported from the white Caucasian group in Chapter 4), from Italy (as reported by Alimonti *et al*., 2009) and according to EURO TERVIHT reference values (Minoia *et al*., 1990).

The overall Linguaglossa study population shows an about 27 and 8 fold increase in geometric mean of urinary manganese compared to the German and the Italian respectively, as reported in Figure 6-8. Manganese levels in urine of the Linguaglossa population (geometric mean 1.70 μg Mn/L) were relatively close to the upper limit set by Minoia (1.90 μg Mn/L) and exceeded the 60th percentile (2.1 μg Mn/L). These values of urinary Mn were close to those reported by Al-Rmalli *et al.* (2011a) in a Bangladeshi community residing in the UK and exposed to an additional Mn source by chewing betel quid(mean: 1.93 μg/L, SD 1.8), compared to the general UK White Caucasian population (0.62 μg Mn/L, SD 0.4).

Urinary nickel of the Linguaglossa study population was higher than the German population (about 11 times higher) and the other Italian population (about 4 times higher). Furthermore, urinary nickel in the Mt. Etna population had a geometric mean that was slightly higher than the upper limit of the reference value for Italy. Values for both urinary Mn and Ni from Table 6-15 are shown in Figure 6-7, highlighting the increased levels in the Mt. Etna population compared to data presented in the literature for Italy and Germany.

Urinary selenium was higher than the German (Heitland and Köster, 2006) and British levels (Chapter 4). However, it was closer to that reported for Italy (Alimonti et *al.*, 2009). Geometric means for cobalt, copper, zinc, cadmium, lead were within the range of reference values for the Italian population (Minoia *et al.*, 1990) and comparable to values reported for Italy (Alimonti *et al*., 2009) and Germany (Heitland and Köster, 2006) .

Table 6-15: Comparison of urinary trace elements levels to literature [µg/L]

	GERMANY[1] *(n=87)*	**UK**[2] *(White, n=23)*	**ITALY**[3] *(n=52)*	**ITALY**[4] *Reference values*	**LINGUAGLOSSA**[5] *(n=49)*
Manganese	0.063 (LOQ-0.71)	NA	0.22 ± 0.10	0.12- 1.90	1.70 (2.1-2.8)
Cobalt	0.18 (0.02–3.3)	NA	0.24 ± 0.18	0.18-0.96	0.4 (0.5-1.9)
Nickel	0.30 (LOQ -7.2)	NA	0.87 ±0.5	0.06-1.74	3.2 (3.3-6.6)
Copper	8 (4 –30)	10.5	12.9 ±7.0	4.20-50	12.6 (14.1-30.3)
Zinc	207 (49–968)	350	356 ± 236	226-846	265 (351-839)
Cadmium	0.17 (0.04–0.56)	NA	0.81 ±0.53	0.38-1.34	0.380 (0.434-1.274)
Thallium	0.069 (LOQ-1.44)	NA	NA	0.07-0.7	0.259 (0.273-0.814)
Lead	0.6 (0.02–4.8)	NA	1.8 ±1.40	12-27	0.992 (1.17-5.70)
Chromium	0.127 (LOQ -1.0)	NA	0.21 ± 0.11	0.04-1.50	0.74 (0.35-1.20)
Arsenic	13 (1 –375)	11.2	NA	2.31-31.3	31.5 (35.0-77.8)
Selenium	12 (3 –60)	18.9	33.5 ± 15.0	2.1-31.3	28.4 (37.3-62.7)

(1) (Heitland and Köster, 2006) geometric mean (range) (2) median for white Caucasians from Chapter 4 corrected by SG, (3)(Alimonti et al., 2009); (4) reference values proposed for the Italian population in the period 1990-2009, from Minoia et. al 1990, lead values are overestimated; (5)geometric mean (60^{th} – 95^{th} percentile).

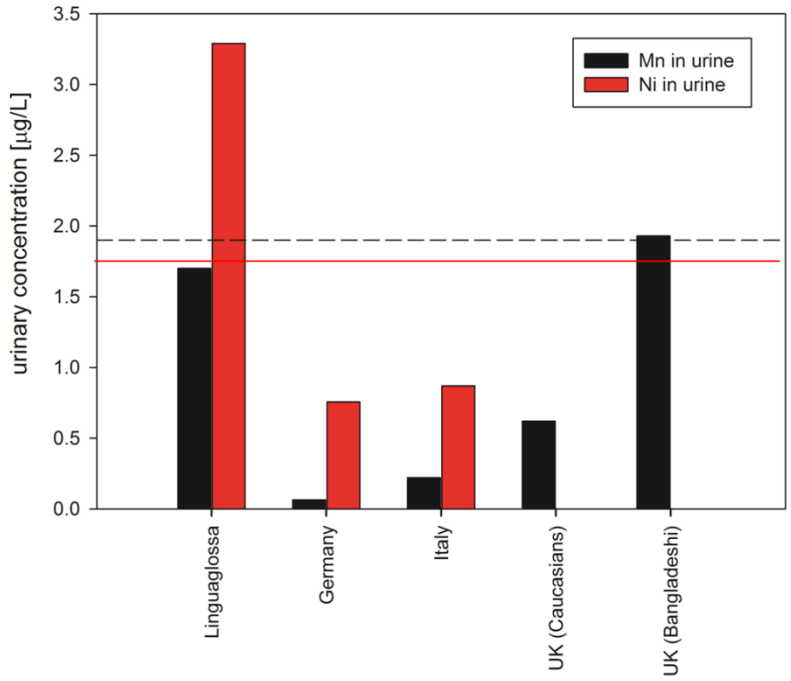


Figure 6-7: Urinary manganese (black bars) and nickel (red bars) for the overall Linguaglossa (*n*=49) compared to a German one (*n*=87) (Heitland and Köster, 2006) an Italian (*n*=52) one (Alimonti et al., 2009). Moreover for manganese urinary levels reported for UK white Caucasians and Bangladeshis are shown (Al-Rmalli et al., 2011). Bars are based on central values reported in table 6-15. Horizontal lines represent the upper limit of the reference values for the italian population for Mn (black dotted) and for Ni (red continuos) as set by (Minoia *et al.,* 1990).

Arsenic was higher in the urine of the Linguaglossa population than that reported for Germany (Heitland and Köster, 2006), and the geometric mean was higher than the upper limit of the reference value. However, as generally known (and largely discussed in the discussion section of Chapter 4), total arsenic in urine is not a good estimation of exposure to inorganic arsenic since it also includes arsenobetaine and other arsenic species that are believed not to have toxicological interest. Consequently, the high levels reported in the general population from Linguaglossa are likely to be due to higher fish consumption in this study group. Fish was in fact quite often stated as one of the foods consumed in the two day food diary completed by the study group.

Baseline levels for trace elements in toenails found in this study for the Mt. Etna population are compared to reference values in Table 6-16 and Figure 6-8. In contrast to urine, there are a smaller number of papers reporting trace elements in toenails. Furthermore, different studies apply different washing procedures that can selectively alter TE content in toenails by leaching some of them. Moreover, different authors use

different statistical parameters and a comparison is not always easy or formally correct. Other populations considered are healthy volunteers from Emilia- Romagna, Northern Italy (Bergomi *et al.,* 2002), who are likely to maintain an Italian diet but are not exposed to the volcanic environment, and an Arab-American population from an industrial part of Detroit (Slotnick *et al.,* 2005). In Figure 6-8 other values for arsenic in toenails of study groups from the UK and the US are considered. The group from Bangladesh is affected by arsenic in drinking water (Kile *et al*., 2005).

Mn levels in the Linguaglossa study group were compatible with the ones reported for the Italian population (Bergomi *et al*., 2002) and from Detroit (Slotnick *et al.,* 2005). With regard to arsenic, the levels were similar to the reported European and American levels, but were drastically different to those from Bangladesh who are known to be exposed to arsenic from drinking water (as discussed in detail in Chapter 2).

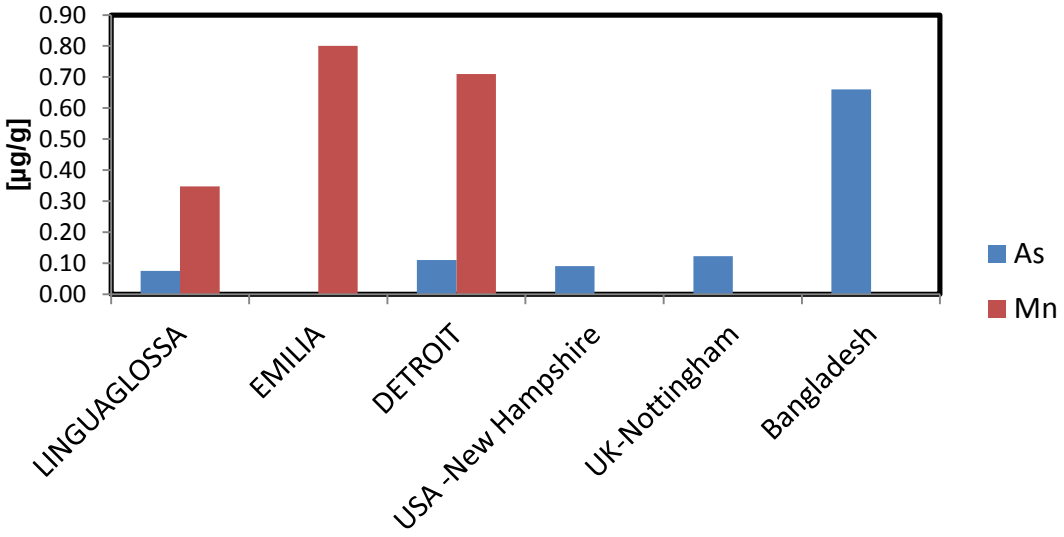


Figure 6-8: Trace element content in toenails of study population from Linguaglossa, Emilia Romagna (Italy) (Bergomi et al., 2002), Detroit (Slotnick et al., 2005), USA -New Hampshire (Karagas et al., 2000), Nottingham (UK) (Button et al., 2009) and Bangladesh arsenic affected area (Kile et al., 2005). Bars represent medians (for Linguaglossa and Italy) for arsenic (blue) and manganese (red).

Table 6-16: Trace element content in toenails of Mt. Etna *vs* the rest of Italy and US [µg/g]

	LINGUAGLOSSA			IT- Emilia			US- Detroit *Arab-Americans*	
	MEDIAN	25th	75th	MEDIAN	25th	75th	MEDIAN	SD
V	0.033	0.019	0.041	NA	NA	NA	0.06	0.06
Mn	0.35	0.23	0.67	0.80	0.42	1.72	0.71	1.09
Fe	14.9	11.3	22.0	22	16	72	NA	NA
Co	0.009	0.004	0.016	NA	NA	NA	0.20	0.76
Ni	0.15	0.08	0.31	NA	NA	NA	37.42	109
Cu	2.96	2.55	3.47	4.24	3.57	6.84	5.28	4.85
Zn	91.4	79.1	103.4	102	92	123	NA	NA
As	0.08	0.06	0.11	0.59	0.53	0.70	0.11	0.22
Se	0.72	0.58	0.83	NA	NA	NA	0.76	0.39
U	0.01	0.01	0.02	NA	NA	NA	NA	NA
Ba	0.45	0.25	0.69	NA	NA	NA	1.37	2.06
Sr	1.02	0.65	1.44	NA	NA	NA	NA	NA

References: IT- Emilia Romagna (Italy) (Bergomi *et al.,* 2002), Detroit (Slotnick *et al*., 2005). Note that Arab Americans from Detroit present an excess of Ni in toenails highlighted by the author.

According to this comparison, Fe was reduced in the toenails of the Mt. Etna group, with about a 1.5 fold decrease in the median and about 3.5 times at the 75th percentile in comparison to the other Italian population from Emilia Romagna. Vanadium and selenium levels were comparable with American values (Slotnick *et al*., 2005). However, this comparison has limitations due to the low recovery obtained for these elements. Cobalt and nickel show a pronounced decrease in the population from Linguaglossa in comparison to the Arab-American group (Slotnick *et al*., 2005). However, this increase could be the result of exposure to industrial chemicals because Slotnick *et al*. (2005) stress the fact that elevated levels of Ni in this population living in an industrial area warrant further investigation.

3.9 Trace element composition of urine and toenails

Figure 6-9 shows scatterplots (toenails *vs* urine concentrations) for some elements. Correlation between urine and toenail trace element concentration was explored by means of a Spearman test. The only significant positive correlation was found for selenium (P=0.024, r=0.348), with no differences seen between MS (red dots) and CNT (green dots). No significant correlation was seen between toenail and urinary trace elemental composition for Mn, Ni, Cu, Zn, As, Pb, Cr and Cd in the overall population.

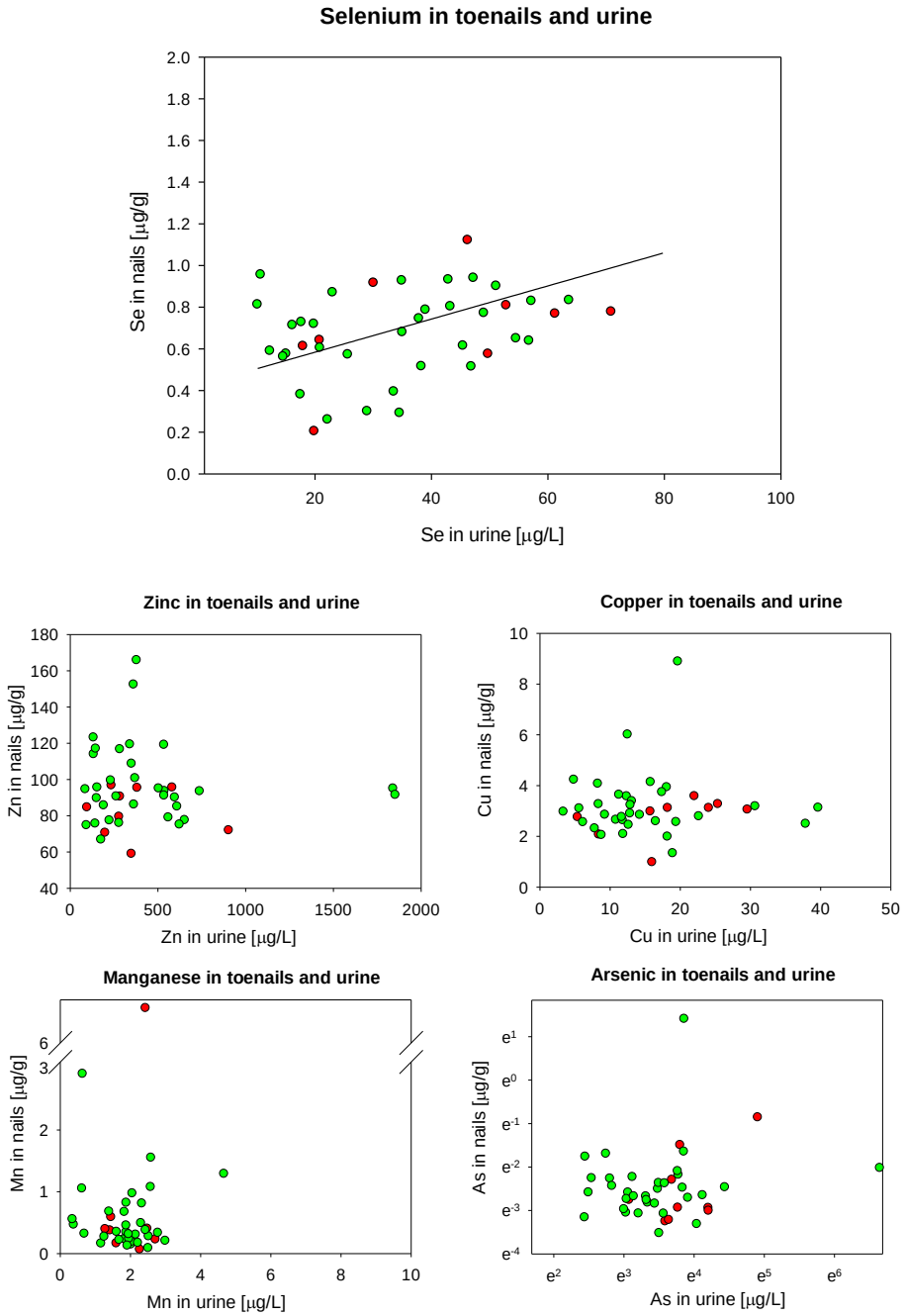


Figure 6-9: scatter plots for selected trace elements in toenails and urine of MS cases (indicated by red dots) and controls (green dots) of Mt. Etna region.

3.10 Toenail to urine ratio

Toenail to urine ratio was calculated for those elements for which data in both bio-samples was available. Figure 6-10 shows relative ratios for cases (MS) and controls (C). For Ni and Cu, a trend of decrease in toenail/urine ratio was seen even though the use of a Mann-Whitney did not reveal a significant difference, with P=0.086 and 0.066 respectively.

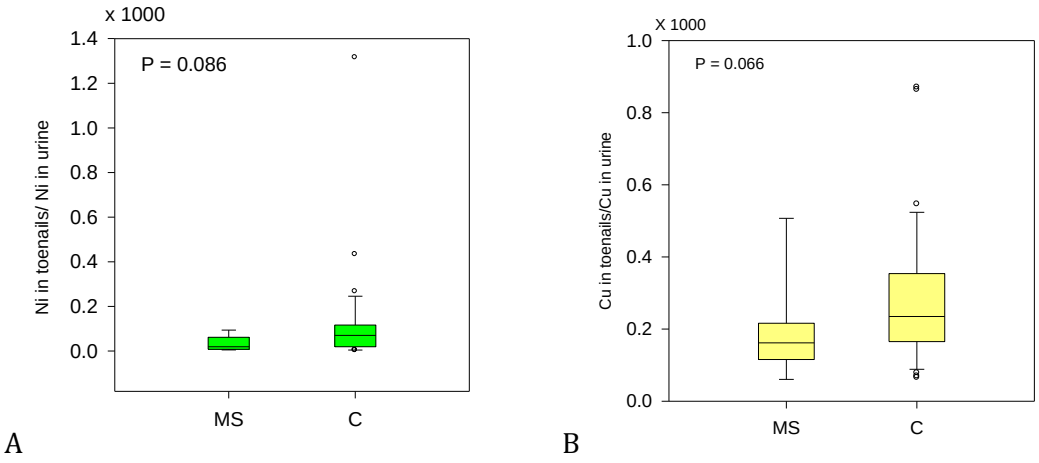


Figure 6-10: nickel toenails to urine ratio for nickel (A) and copper (B).

3.11 Trace element correlations in urine

To assess interactions of trace elements analysed in urine a Spearman Rank Order Correlation was performed using SPSS. Significant ($p < 0.005$) correlation coefficients are reported in Table 6-17. Couples with a correlation coefficient higher than 0.7 are reported in bold. All correlations were positive. The strongest correlation was that between Cu and Ni (0.870), followed by Cu and Se (0.801).

Table 6-17: Correlation matrix for trace elements in urine

	Mn	Co	Ni	Cu	Zn	Cd	Tl	Pb	Cr	As	Se
Mn	1	0.328	0.489	0.542	0.373	0.349	0.425	0.304	0.511	0.423	0.468
Co		1	**0.799**	**0.707**	0.599	0.649	0.448		0.665	0.405	0.658
Ni			1	**0.870**	**0.765**	**0.741**	0.628	0.582	**0.779**	0.405	**0.707**
Cu				1	**0.755**	**0.794**	**0.705**	0.553	**0.702**	0.514	**0.801**
Zn					1	**0.707**	0.494	0.446	0.618	0.365	0.629
Cd						1	0.541	0.361	0.57	0.33	0.67
Tl							1	0.371	0.609	0.462	**0.708**
Pb								1	0.441		0.415
Cr									1	0.327	0.674
As										1	0.541
Se											1

4. Discussion

The relationship between exposure to trace elements and Multiple Sclerosis in the cluster of Linguaglossa in the Mt. Etna region, was investigated in this chapter, for the first time. Moreover, diet, local food consumption and general habits were investigated as risk factors for MS in this cluster.

In addition, for the first time baseline levels of urinary and toenails trace elements for a Sicilian population are also presented. To the best of this author's knowledge nothing has been published for the Mt. Etna region in this sense apart from levels of total mercury in hair in a study group from Catania compared to a group for Augusta (SR, IT) (Ferrante *et al.*, 2004).

Among the trace elements analysed in toenails, nickel showed a trend of reduction in MS patients (P=0.081) in comparison to healthy controls from Linguaglossa. This resulted in a lower toenail to urine ratio for Ni in MS than in CNT. Furthermore, urinary nickel in the overall study population from Linguaglossa was about 4 times higher than the Italian values (Alimonti *et al.,* 2009) and about 11 times higher than the German values (Heitland and Köster, 2006). Once nickel is in the blood, it is predominantly extracted by the kidneys with a first-order kinetic reaction (Christensen and Lagesson, 1981), and is found in the urine regardless of the route of exposure (ATSDR, 2005).

The elevated levels of nickel in urine of the overall population from the volcanic region of Linguaglossa compared to Italy and Germany could be the possible result of exposure to higher levels of environmental nickel, including *via* diet, as shown by Fallico and Ferrante

(1999). The possible decreased toenail/urine Ni ratio in MS patients might be related to a lower storage of nickel in the toenails of MS in respect to CNT. Behind this, a different chemical form, or the presence of proteins bound to Ni in MS, could be the triggers for decreased nail storage of Ni and possible accumulation in the serum or other regions of the body such as the lymph nodes or CSF (Melo *et al.*, 2003). To support this hypothesis, Alimonti (2007b) found an increase of Ni in serum in 60 MS patients compared with the serum of 124 healthy controls.

Nickel is a metallic element naturally present in the Earth's crust and along with its compounds, is widely used in modern industry (Keim, 1990). Human exposure to nickel occurs primarily via inhalation and ingestion and it is known that exposure to nickel compounds can have adverse effects on human health (allergy, lung fibrosis, cardiovascular and kidney diseases and carcinogenic activity) (Denkhaus and Salnikow, 2002). *In vitro* evaluation of the toxicity induced by nickel soluble and particulate forms in human epithelial cells shows that exposure to $NiCl_2$ and Ni particles result in a disruption of the epithelial barrier function. Ni compounds induce oxidative stress associated with ROS formation and up-regulation of the stress-inducible genes, metallothionein 1X (MT1X), heat shock protein 70 (HSP70), heme oxygenase-1 (HMOX-1), and gamma-glutamylcysteine synthetase (γGCS) (Forti *et al.*, 2011). In its soluble ($NiCl_2$) and insoluble (Ni_3S_2) species, nickel produces low but measurable effects on cells, inducing free radical production (Denkhaus and Salnikow, 2002). $NiCl_2$ induces oxidative stress and genotoxicity in human lymphocytes (Chen *et al.*, 2003a). Ni induces a dramatic depletion of the antioxidant glutathione (GSH) in hepatic cells in both *in vivo* and *in vitro* studies (Denkhaus and Salnikow, 2002). Additionally, $NiCl_2$ exposure in cultured human cells up-regulates intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial leukocyte adhesion molecule-1 (ELAM-1, E-selectin) (Goebeler *et al.*, 1993, Denkhaus and Salnikow, 2002). It is noteworthy that these endothelial surface molecules play a central role in the MS pathogenesis (Matsuda *et al.*, 1995). In fact, as a result of the inflammatory reactions in MS plaques, various cytokines can be detected in the CSF of MS sufferers. Pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) or interferon gamma (IFN-γ) up-regulate expression of ICAM in the CSF of MS patients (Engelhardt and Ransohoff, 2005). The significance of ICAM as a measure of disease activity has been extensively studied in patients with MS (Lee and Benveniste, 1999). A correlation of ICAM-1 levels in the CSF, along with relapse activity, active MRI lesions, dysfunction of the blood-brain barrier and myelin-basic protein (MBP) levels have

been demonstrated (Dore-Duffy *et al.*, 1995, Lee and Benveniste, 1999, McDonnell *et al.*, 1998, Rieckmann *et al.*, 1993, Acar *et al.*, 2005, Alves-Leon *et al.*, 2001). It should be noted that Natalizumab, a current available therapy for the treatment of Relapsing Remitting form of MS, is a humanized monoclonal antibody that binds to α-4 integrin molecule, preventing the extravasation of T and B cells into the CNS, and consequently reducing inflammatory immune reactions in lesions of MS patients (Ransohoff, 2007; Hutchinson, 2010).

The general population is mainly exposed to nickel through food intake, although Ni can also be inhaled from the air. Data on nickel content in food the Mt. Etna region (Fallico and Ferrante, 1999) showed an average weekly intake of 86.8 ± 5.4 μg/kg b.w. and this represents 74.4 % of the Upper Limit set by the Institute of Medicine (Food and Nutrition Board, 2001). Groundwater samples from Linguaglossa did not show an excess of Ni according to measurements (0.42 ± 1.34 ppb) (data reported in table A6-5 of the Appendix of this chapter). A possible additive source of nickel exposure in the local population comes from the emission of nickel in the soil or in the volcanic plume; Mt. Etna emits about 100 tons/year of nickel (Calabrese, 2008). The most plausible explanation for the increase in nickel levels in urine of the Mt. Etna group could be the combined exposure through the dietary route, volcanic dust and soil. The increasing incidence of MS over time has been recently reported in several geographic areas not related to the presence of an active volcano. However, Ni environmental exposure and, in particular, the environmental pollution by nickel and its by-products related to the high consumption of nickel-containing products in modern industry could play a role. In fact, other hidden sources of human exposure to nickel are present in everyday life; there are about 3000 nickel-containing alloys in daily use[21] including jewellery, stainless steel objects and devices in the body. A Danish study has demonstrated that 19.3% hair clasps, 14.8% earrings, and 12.9% necklaces intended for adult women sold in Danish shops and vendors release an excessive amount of nickel (Thyssen *et al.*, 2009). Release of nickel was also found in products sold in children's clothing stores.

The data presented in this thesis suggested an overall higher level of Ni exposure in the Linguaglossa population; Ni levels in the whole sample population were higher than those reported in the Italian population. Moreover, there might be a greater nail/urine ratio of

[21] http://www.nickelinstitute.org/index.cfm/ci_id/10172/la_id/1.htm

Ni among MS cases with respect to the CNT subjects. Taking into account the small sample size, the trend seen for Ni could be confirmed in a greater scale study. Furthermore, a major limitation in the interpretation of the findings is related to the retrospective nature of the study that does not allow for the exclusion of a possible reverse causality, i.e. that increased Ni ratio is not the cause, but the consequence of the disease.

Interviewed volunteers from Mt. Etna revealed a widespread use of groundwater for drinking and cooking purposes and the consumption of locally grown food. Questionnaire data show no significant differences in the dietary and other factors (smoking, sports, BMI, use of bottled/tap/well water, tea, coffee, alcohol consumption) between the MS cases and controls. In general, confidence intervals were very broad and this could be due to the small number of cases.

Urine and toenails from both MS cases and controls from Linguaglossa did not show a significant difference in the trace element composition, except for a slight decrease in urinary chromium levels in MS patients after specific gravity correction ($p= 0.045$). However, toenails of MS patients appeared to generally be less rich in trace elements than the controls.

Other differences noticed in the urine of the Mt. Etna group were related to the increase in manganese. Analysis of trace element levels including Mn in groundwater samples from public fountains in the region of Linguaglossa and surroundings was carried out. This revealed that whilst Mn levels in the Etna region were found to be as high as 1810 ppb (Roccaro *et al.*, 2007a), the levels in the specific region in this study were low (0.09 ± 0.81 ppb). This great variation from point to point in the Mt. Etna groundwaters has been previously addressed (Giammanco *et al.*, 1996). One of the possible sources of Mn is diet. However, the estimated weekly Mn intake in the city of Catania is 1283.3 ± 38.3 μg/kg b.w. (Fallico and Ferrante, 1999) accounting for just 19% of the UL set by IOM. The reason for such an increase in urinary Mn therefore remains unexplained.

At the 60th percentile, urinary selenium (37.3 μg/L) of the Linguaglossa population exceeded the upper limit of the reference value for Italian population (31.3 μg/L). Excluding drinking water as a reason (Se levels in the local region is 0.85 ± 0.11 μg/L), the increase in urinary selenium in the Mt. Etna region could be the result of different factors amongst which could be diet or environmental source. The correlation between urinary Se and toenail Se suggests a relationship between exposure, excretion (urine) and storage

(toenail) and therefore the presence of a stable exposure source is very likely. This could be related to a dietary source such as the regular intake of staple foods that are wheat-based products (pasta and bread) or water intake (from both drinking and cooking foods). This possibly reflects the different selenium intake between the Italian diet compared to the German and British diets. In fact, wheat grown in the South-East of Sicily has selenium levels ranging from 80-120 ppb (Spadoni *et al.*, 2007). The selenium dietary exposure for Italy is estimated to be 103.6 μg /day (Lombardi-Boccia *et al.*, 2003) while Rayman reported a daily intake of Se < 50 μg /day for both UK and Germany (Rayman, 2005) (see graph in Figure 6A-1 in the Appendix to this chapter). Cereals contribute to the 15% of Se daily intake and meat and meat products account for 20% (Lombardi-Boccia *et al.*, 2003). Fish accounts for 20% as well according to data from Lombardi-Boccia *et al.* (2003). Wheat is a staple food for the Italian diet in the form of bread and pasta. So it is not surprising that the Italian group has higher urinary selenium than the German or UK study groups. However, in the case of the Mt Etna study group, vegetables, garlic, nuts and almonds could contribute to the increase in urinary Se with respect to the other Italian groups considered here (Alimonti *et al*., 2009). In contrast to selenium, the sources of the other trace elements determined in this study are so varied that no correlation is observed because the urine gives a recent exposure and toenails provide a longer term exposure. Consequently, a very rapid change in the day to day diet will cause the urine levels to be very different from that of toenails, which provide a more time averaged picture.

The increase in urinary arsenic seen in the Mt. Etna population compared to German, British and Italian referred values as detailed in Table 6-15 is likely to be the result of sea fish consumption. As already described, total urinary levels of arsenic are not suitable for risk assessment or as a general estimate of exposure because of the contribution from arsenobetaine and cationic compounds that are so far thought to not be harmful for human health. In this study, the volunteers were not asked to refrain from eating fish prior to providing urine samples, but a two day food diary was kept. It is likely that the higher arsenic levels in the Mt. Etna population are related to high incidence of fish consumption because the region is close to the sea and seafood consumption is common in this population, as is evident from the questionnaire data. This is also confirmed by the absence of any correlation between urinary and toenail arsenic.

Finally, the revealed presence of ketones in the urine of one volunteer affected by MS could be the result of catabolism of fatty acids coming from degradation of the myelin

sheath. A metabolomic analysis of urine of MS (data not presented in this thesis) has shown an increase in acetoacetate compared to controls.

5. Study limitation

Clusters are important sources of information for researchers and their study gives the chance of an insight into possible genetic or environmental alterations triggering the pathology of a disease. However, limitations arise from this approach. Firstly the time factor, i.e. the delay between the time of exposure and the onset of the disease and the sampling moment. The analysis of toenails and urine as biomarkers of exposure allows the detection of current and recent exposure to contaminants. MS induction in this cluster of patients probably happened several years before sampling since it is believed that adolescence is the time of induction to MS. Another limitation of this study is the inevitability of a small number of cases to constitute the sample size. This limits the ability to differentiate between an excess of etiologic interest and a chance occurrence; epidemiologists have long known that evaluating perceived clusters is rarely fruitful for identifying an etiologic agent (Williamson and Henry, 2004).

6. Conclusions

This is the first study to investigate the possible relationship between trace element exposure and multiple sclerosis in Linguaglossa on the Mt. Etna, an active volcanic site.

The role of diet and other risk factors were considered by means of a questionnaire. Although no significant difference in the trace elemental composition of urine of MS cases and controls was observed for most of the elements studied, the study population from Linguaglossa showed a characteristic increase in the level of nickel, manganese and selenium that could be related to peculiar geochemical conditions in the volcanic site, which could in turn influence the food chain. The possible involvement of nickel in the immune reaction that triggers the development of MS is here hypothesized for the first time.

7. Future Perspectives

Larger studies on the possible role of Ni on MS, and in general of trace elements, should be performed. In order to add further evidence on the possible role on Ni in MS pathogenesis,

a study was carryied out to evaluate the Ni level on CSF of new onset cases of MS (Chapter 7).

Despite the fact that the epidemiology of MS is one of the most active research fields worldwide, the cause of this disease is still unknown. From this point of view, every new hypothesis is welcome and should be investigated by employing innovative research techniques. This can be best achieved through collaboration across disciplines that will be able to effectively guide research efforts for determining the aetiology of Multiple Sclerosis.

8. Acknowledgments

I would like to thank all the volunteers affected by Multiple Sclerosis and the controls from Linguaglossa. The section of Linguaglossa of the Associazione Italiana Malati Sclerosi Multipla (AIMS) is to be acknowledged for the help and support in recruitment. I would like to thank Prof. Alessandra Nicoletti for collaboration and Prof. Franco Patti, Dr. Salvo Lo Fermo, Prof. M. Zappia, Elisa Bruno, Silvia Messina and Graziella Quattrocchi from the Department of Neurosciences of the University of Catania (Italy). I want also to thank Jackie Morton from the Health and Safety Laboratory for the collaboration.

Chapter 7

ARSENIC AND OTHER TRACE ELEMENTS IN CEREBROSPINAL FLUID OF PATIENTS AFFECTED BY MULTIPLE SCLEROSIS

1. Introduction

Inorganic arsenic (iAs) is a non-threshold class I carcinogenic agent (IARC, 2004) causing cancer of lungs, skin and bladder (Chen *et al.,* 1985) and responsible for about 20% of deaths in Bangladesh (Argos *et al.,* 2010). Furthermore, arsenic presents non carcinogenic effects, among which is neurotoxicity. Chronic exposure to arsenic has been related to effects on the central nervous system (CNS), such as alteration in learning memory and concentration, decrease in verbal IQ and hearing alteration (Bencko and Symon, 1977). Evidence for neuropathy induced by chronic exposure to arsenic has been reported in Bangladesh (Rahman *et al.,* 2001). Additionally, an increased arsenic exposure was associated with subclinical sensory neuropathy in Bangladesh (Hafeman *et al*., 2005). More recently, urinary As was found to be significantly negatively associated with verbal comprehension scores and neurobehavioral function in children from Bangladesh (Wasserman *et al.,* 2011). Arsenic metabolites have been found in the brains of rodents after a massive oral administration of inorganic arsenic, and a dose-related accumulation of As species was demonstrated in all brain regions and especially in the pituitary gland (Sanchez-Pena *et al*., 2010). Plasma cholinesterase activity has been shown to be inversely

correlated to arsenic exposure through drinking water in the human population in Bangladesh (Karim *et al*., 2010).

Arsenic exists in the natural environment as different chemical species (explained in Chapters 1 and 2), that exhibit a range of diverse toxicological properties. Arsenic occurs in water, soil, air and food and especially rice (Meharg *et al.*, 2009b) as a result of geogenic (Winkel *et al*., 2011; Nickson *et al*., 1998) and/or anthropogenic processes (Williams *et al.*, 2005b). Organic arsenic, mainly in the form of arsenobetaine, is largely present in sea fish and marginally in poultry and represents an additive dietary source of arsenic of minor toxicological interest. Once arsenic is ingested it reaches the gastrointestinal tract, and is absorbed to variable extents depending on the chemical species, which modulates bioavailability. From the blood, arsenic reaches its target organs.

Other metals are known to have harmful effects of nervous system. Lead has neurological effects on adults and children the most serious of which is encephalopathy (ASTDR, 2007). Aluminium (Polizzi *et al*., 2002), manganese (Bowler *et al*., 2006) nickel (He *et al*., 2011) and cadmium (Minami *et al*., 2001) have been reported to be neurotoxic. Conversely, essential trace elements such as selenium, copper and zinc have a physiochemical role in the human body and in the CNS. However, an imbalance of their concentration in bio-fluids has been reported associated with neurological diseases (Forte *et al*., 2004) (Gerhardsson *et al.,* 2008), including Multiple Sclerosis (Melo *et al.,* 2003; Gellein *et al.,* 2008).

The CNS and the cerebrospinal fluid (CSF) are generally protected from toxic elements - such as arsenic, lead, mercury and cadmium - present in the blood by the lateral choroid plexus of the blood-brain barrier, which is capable of sequestering metals (Zheng *et al.,* 1991). Nevertheless, it has been demonstrated that in patients with an oral administration of arsenic trioxide (10 mg/day) as a therapy for meningeal relapse acute promyelocytic leukemia (APL), inorganic arsenic is able to significantly penetrate into the CSF, showing a good correlation with As plasma levels (Au *et al.,* 2006). Only one study has reported arsenic speciation in the CSF (Kiguchi *et al.,* 2010), from three patients affected by APL before and after arsenic administration, with very little information about the method of arsenic speciation and peak assignment. Therefore, very little is known on the chemical speciation of arsenic in the CSF of both healthy and sensitive groups, such as people affected by neurological conditions. In general, studies that quantify total trace elements in

CSF are rare (Gellein *et al*., 2008; Alimonti *et al.,* 2007a; Köksaldi *et al.,* 2008; Speziali and Di Casa, 2009). This is partially due to the difficulty of sample collection. There is an urgent need for further studies on CSF, trace elements and arsenic speciation profile of the general population (Michalke and Nischwitz, 2010).

As reported in Chapter 2, Multiple Sclerosis (MS) is an autoimmune disorder affecting 2-2.5 million people worldwide. MS is characterised by an auto-immune reaction *versus* the myelin sheet of neurons and this eventually results in neural loss and formation of sclerotic plaques that are the hallmark of the disease. A few studies have reported trace elements in CSF of MS patients, providing sometimes controversial data on the imbalance of manganese, copper and other elements (Melo *et al.,* 2003; Gellein *et al*., 2008). To the best of this author's knowledge, no data have been published for arsenic content in a cohort of patients affected by Multiple Sclerosis.

Post mortem studies on MS reveal the pathological status of the blood–brain barrier. Lesions were found in acute and secondary progressive forms of MS. In addition, they demonstrate a tight junction disruption in MS and a positive association with evidence of *ante mortem* serum protein leakage across the blood–brain barrier (Plumb *et al*., 2002; Kirk *et al*., 2003).

The present study reports levels of 11 toxic and essential trace elements (aluminium, manganese, cobalt, nickel, copper, zinc, molybdenum, cadmium, lead, arsenic and selenium) in CSF from 36 MS patients (MS) along with 13 controls (CNT). Controls were selected because not affected by any infectious disease, or other neurological disease such as Amiotrophic Lateral Sclerosis, Parkinson's disease and Alzheimer' s disease. Controls were mainly affected by migraines. The fact that non-healthy controls were sampled is to ethically justify the impact and the risk connected with the sampling procedure. Quantification of elements was performed by mean of ICP-MS.

2. Sampling and population description

The study was approved by De Montfort University's Faculty of Health and Life Sciences' Research Ethics Committee and previously by the Ethics Committee of the University of Catania. Sampling was conducted under ethical standards.

CSF samples were collected at the hospital of the Policlinico of the University of Catania (Sicily, Italy) by trained specialist doctors using lumbar puncture, which was performed

by inserting the needle between the fourth and fifth lumbar vertebrae. The study participants consisted of MS patients (MS) (*n*=36) affected by multiple sclerosis, generally during their first visits to the clinic, along with 13 controls (CNT).

After collection, CSF was quickly transferred into transparent plastic vials, observed to ensure the absence of blood contamination, and if clear, selected for further analysis. Part of the CSF was analysed for albumin and total protein content and other clinical analysis at Policlinico. Another aliquot was frozen at -80°C and kept frozen until the day of analysis.

Blood plasma samples were collected by endovenous puncture in vials containing heparin. After centrifugation at 4 rpm for four minutes, the supernatant was transferred in plastic tubes and frozen at -80° C.

Frozen samples were transported in dry ice to De Montfort University, Leicester (UK) and kept at -80°C till the day of the analysis at the Health and Safety Laboratory, Buxton (UK). Multiple sclerosis patients were mainly females (73.7%) while 47.3% of controls were females.

3. Methods

3.1 Materials

Multi-element standard solutions were prepared from a 100mg/L multi-element standard solution (Primar, BDH, Poole, UK) in 1% v/v nitric acid (Romil Ltd, Cambridge, UK). Single calibrant standards used included arsenic, selenium, chromium and mercury, made from 1000 mg/L stock ICP-MS standards (BDH, Poole, UK). Arsenic, chromium and selenium were analysed in CCT mode. Single ICP-MS standards used as internal standards were germanium, yttrium, rhodium, indium, platinum and gallium. Also gold was used as an additive in mercury analysis (all 1000 mg/L ICP-MS standards from BDH, Poole, UK). Ethylenediaminetetraacetic acid (EDTA) was from Fisher Scientific, Loughborough, UK. Butan-1-ol (analytical grade), Triton X-100 and ammonia were from Fisher Scientific, Loughborough, UK. External quality control samples were Clinchek level 1, BR level 1 69131 SN serum level 1 lot n. 0608414.

3.2 Preparation and analysis of trace elements in CSF

All biological samples were defrosted at room temperature and mixed on sample rollers for a minimum of 20 minutes. In each analysis the biological sample was diluted at the same dilution as the quality controls samples.

The CSF was spun in a centrifuge at 13000 rev/min for 5 minutes before pipetting.

The calibration range for Al, Mn, Co, Ni, Cu, Zn, Mo, Cd, Tl, Pb was 0.2-20 µg/L. The CSF samples were diluted 1 in 25 if enough sample was available. Otherwise, it was diluted 1 in 40 or 1 in 50. Diluents consisting of 0.1% m/v EDTA, 1% v/v HNO_3, 10 µg/L internal standards (Ge, Y, Rh, In, Pt), 1 mg/L gold (Au) standard. Three CSF samples were spiked at a final concentration of 2.5 µg/L. Three replicate measurements per sample and 50 sweeps were used.

For As, Se and Cr for which a collision cell technology (CCT) was used, the calibration range was 0.2-20 µg/L, CSF diluted 1 in 25. Spikes, diluent and quality controls (QCs) were prepared as detailed before.

The analysis was carried out using ICP-MS (X7 Series 2 ICP-MS, Thermo-Fisher Scientific, Hemel Hempstead, UK). The instrument conditions used direct nebulisation in normal mode with optimised conditions. Extraction voltage was typically -100V, Rf Power 1400W, focus voltage 12.0V and nebuliser gas flow rate (using a Burgener Miramist nebuliser) 0.83 L/min. The instrument was tuned-on on a daily basis to ensure optimisation. In CCT mode the collision gas was 7% hydrogen in helium and the flow rate was typically 3.5 mL/min.

Total protein and albumin analysis were performed by the Central Laboratory of the Policlinico of Catania, according to routine bio-clinical methods.

4. Results

4.1 Quality checks

The full list of analysed isotopes and quality checks is in Table 7-1. A 2 µg/L multi-elemental standard was measured 9 times all along the run, showing a stable signal (% RSD < 3 for Al, Co, Ni, Cu, Mo, Cd, Pb, Cr, As, Se) and slightly higher for Zn (7.5).

A certified reference material for trace elements in CSF is not commercially available, therefore in this study we used serum for quality controls. Spiking of trace elements at known concentrations was done on CSF samples for which a higher volume was available.

Recoveries and limits of detection (LOD) obtained are shown in Table 7-1. Recoveries were generally acceptable, except for Zn (37% in serum) that however showed 81% recovery in spiked CSF.

4.2 Total trace elements in CSF samples

A total of 49 CSF samples were analysed by ICP-MS for the 11 trace elements. Descriptive statistics for trace elements in CSF for both MS and controls are shown in Table 7-2. Since the variables were not normally distributed, univariate statistics on natural logarithm (ln) transformed data were used to assess the presence of any significant difference in MS versus controls. Results of a t-test are reported in Table 7-3 for significant features. The trace element profile in CSF of patients affected by MS was different from controls.

Table 7-1: Quality check for elements analysed in CSF

Element	Isotope	LOD [µg/L]	% REC SR[1]	% REC spk CSF[2]
Al	27	0.722	107	98
Mn	55	0.320	101	97
Co	59	0.018	98	98
Ni	60	0.239	93	95
Cu	65	1.127	64	91
Zn	66	8.15	37	81
Mo	95	0.105	109	105
Cd	111	0.021	97	92
Pb	208	0.044	101	93
As	75	1.25	118	109
Se	78	0.31	103	92

(1)%REC SR: is percentage recovery calculated as 100 *measured value/expected value using serum reference material; (2) % REC spk CSF: is percentage recovery calculated as 100 *measured value/ expected value considering spiked samples.

Table 7-2: Descriptive statistics for trace elemental composition of CSF in 38 volunteers: 13 controls (CNT) and 36 MS cases from this study [µg/L]

CNT (N=13)	Al	Mn	Co	Ni	Cu	Zn	Cd	Pb	As	Se	Mo
mean	8.1	1.6	0.096	2.5	15.1	9.0	0.016	0.19	<LOD	9.5	0.36
st dev	14.8	0.7	0.038	1.4	4.0	<LOD	0.016	0.17	<LOD	18.5	0.24
median	2.0	1.5	0.083	1.9	16.0	<LOD	<LOD	0.10	<LOD	2.9	0.34
60th	2.2	1.5	0.090	2.0	17.0	<LOD	<LOD	0.12	<LOD	3.4	0.35
90th	14.7	2.5	0.157	3.9	20.4	18.6	0.018	0.46	<LOD	15.7	0.42
MS (N=36)	**Al**	**Mn**	**Co**	**Ni**	**Cu**	**Zn**	**Cd**	**Pb**	**As**	**Se**	**Mo**
mean	14.7	2.0	0.112	4.0	19.0	27.7	0.083	0.44	6.5	6.6	0.55
st dev	19.5	1.4	0.075	3.6	9.9	11.9	0.252	0.36	2.1	18.2	0.38
median	5.1	1.4	0.083	2.4	16.5	25.1	0.026	0.35	7.1	1.9	0.42
60th	8.7	2.1	0.098	3.5	18.1	27.2	0.030	0.54	7.2	2.3	0.54
90th	38.1	3.3	0.231	7.7	33.1	43.8	0.098	0.82	8.8	4.4	0.83

Table 7-3: Results of the t-test performed on ln concentrations of elements in CSF of MS and CNT

Element	P value
Al	**0.021**
Mn	0.485
Co	0.951
Ni	0.118
Cu	0.198
Zn	**<0.001**
Cd	**0.003**
Pb	**0.021**
As	**<0.001**
Mo	**0.016**
Se	0.104

The most pronounced differences between CSF from MS and CNT were seen for arsenic and zinc (for both $p<0.001$), that were significantly increased in MS compared to CNT. On the median zinc was about 4 times higher in MS than in controls. Furthermore, aluminium, cadmium, lead and molybdenum were significantly increased in CSF of MS than in CNT. Selenium conversely showed an opposite trend, being higher in CNT than in MS, although this was not statistically significant. Box plots of some elements screened in CSF are represented in Figure 7-1.

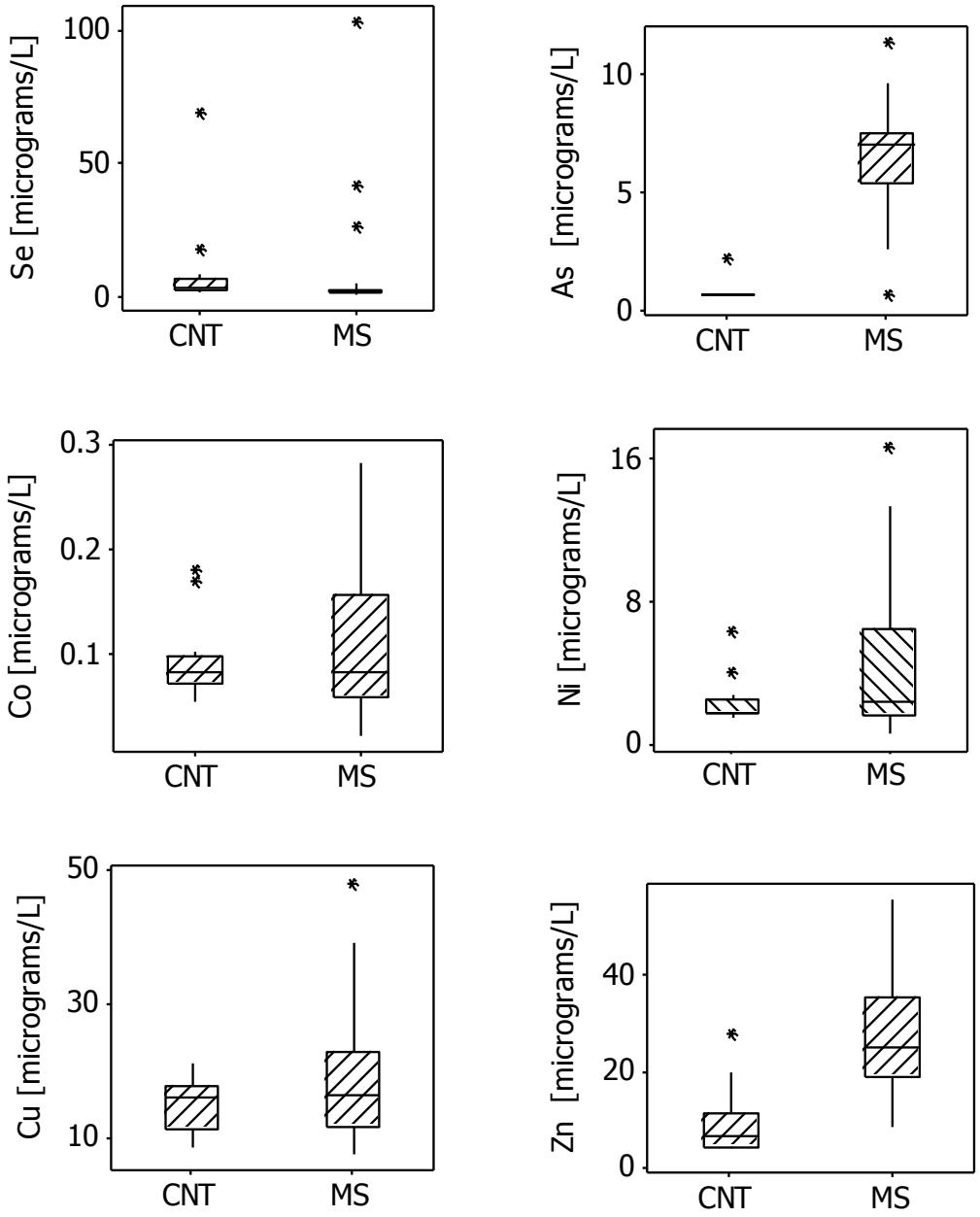


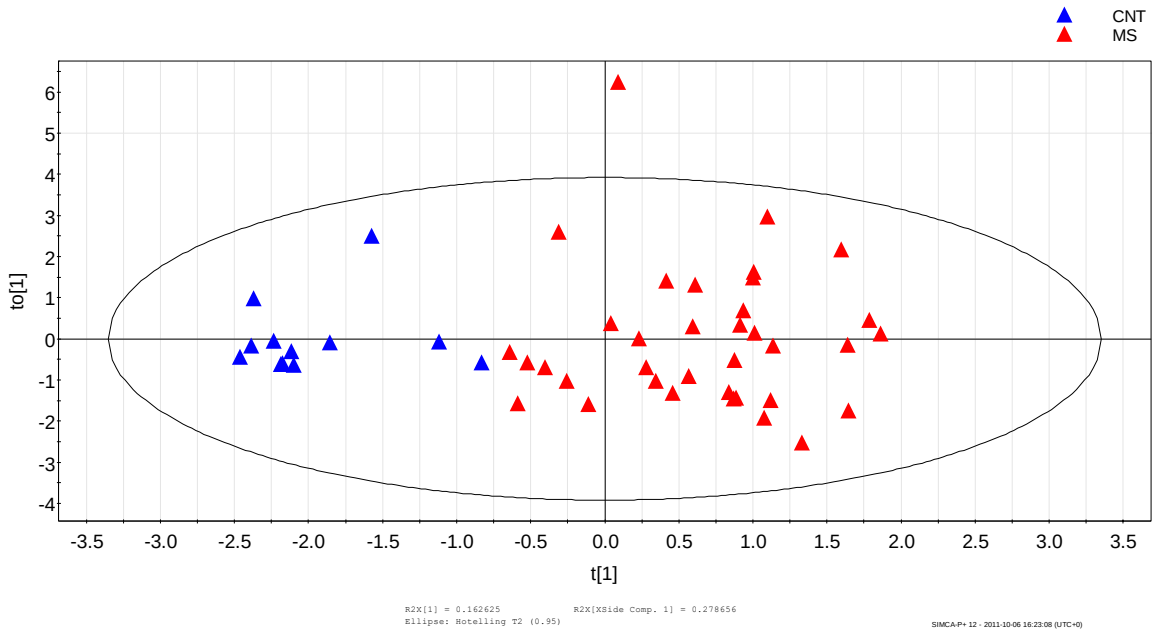
Figure 7-1: Box plots showing trace elemental composition for CSF of 13 controls (CNT) and 36 cases affected by multiple sclerosis (MS) .

4.3 Arsenic increases in CSF of MS plays the main role in separation

A multivariate approach was used to investigate trends in the overall CSF elemental composition of MS and controls from this study. A supervised multivariate approach such as Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) allows the simultaneous comparison of the entire elemental profile of CSF rather than the single element, and allows for the identification of selective variation in the dataset, driving the separation between MS and CNT. By means of OPLS-DA, a model was fitted in SimcaP+. The model showed good $R^2Y = 0.7$ (estimated model explanation) and $Q^2 = 0.6$ (estimated model prediction). As shown in Figure 7-2, a certain level of separation was obtained on the t[1] axis between CSF of MS (red triangles) and CNT (blue triangles). The t0 [1] axis shows the intra-group variability. It is evident that inter-individual variability of the trace elemental profile was more affected in MS than in CNT. The main role in driving the inter group variation was played by an increase in As, Zn and Pb in CSF of MS (see Figure 7-2 B) compared to CNT. Interestingly, a decrease in Se levels in the CSF for MS patients emerged using this approach, even if the variation was too big to consider it as a significant feature driving the separation.

It is noteworthy that a partial agreement between the multivariate and the univariate approach is seen, with an increase in As, Zn and Pb of CSF of MS recognised as important features by both approaches.

A)



B)

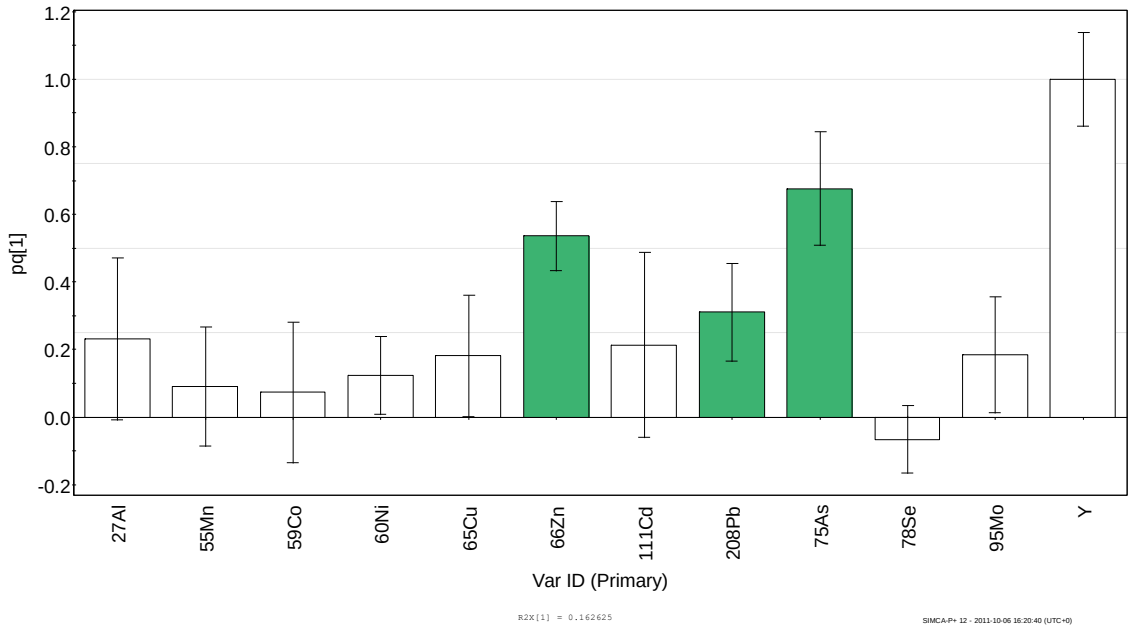


Figure 7-2 A): OPLS-DA model fit for CSF composition of MS (red triangles) and CNT (blue triangles); B) important features identified for the separation include Zn, Pb and As.

4.4 Arsenic positively correlates to zinc and negatively to selenium in CSF of MS

A correlation analysis (by Pearson test on ln transformed elemental concentrations of the overall study population), showed that some elements were strongly correlated to each other (as presented in Table 7-4). The only negative correlation was present between arsenic and selenium. Some of these correlations are presented as scatter plots in Figure 7-3.

Table 7-4: Correlation by Pearson test on ln concentration in CSF of the overall study population (only significant relations are presented).

Element 1	Element 2	P value	Person Correlation
Co	Mn	<0.001	0.784
Zn	Cd	<0.001	0.530
Pb	Cd	<0.001	0.444
As	Zn	<0.001	0.671
Co	Ni	<0.001	0.775
Ni	Mn	<0.001	0.671
Al	Zn	<0.001	0.509
Mo	Cu	<0.001	0.533
Mo	Zn	0.002	0.438
As	Se	0.001	-0.460
Mo	Mn	0.002	0.438
Cu	Mn	0.004	0.409
Mo	Cd	0.025	0.320
Cd	Al	0.001	0.476
Al	As	0.051	0.281
Pb	Cu	0.018	0.337
As	Cd	0.020	0.331
Zn	Pb	0.011	0.359

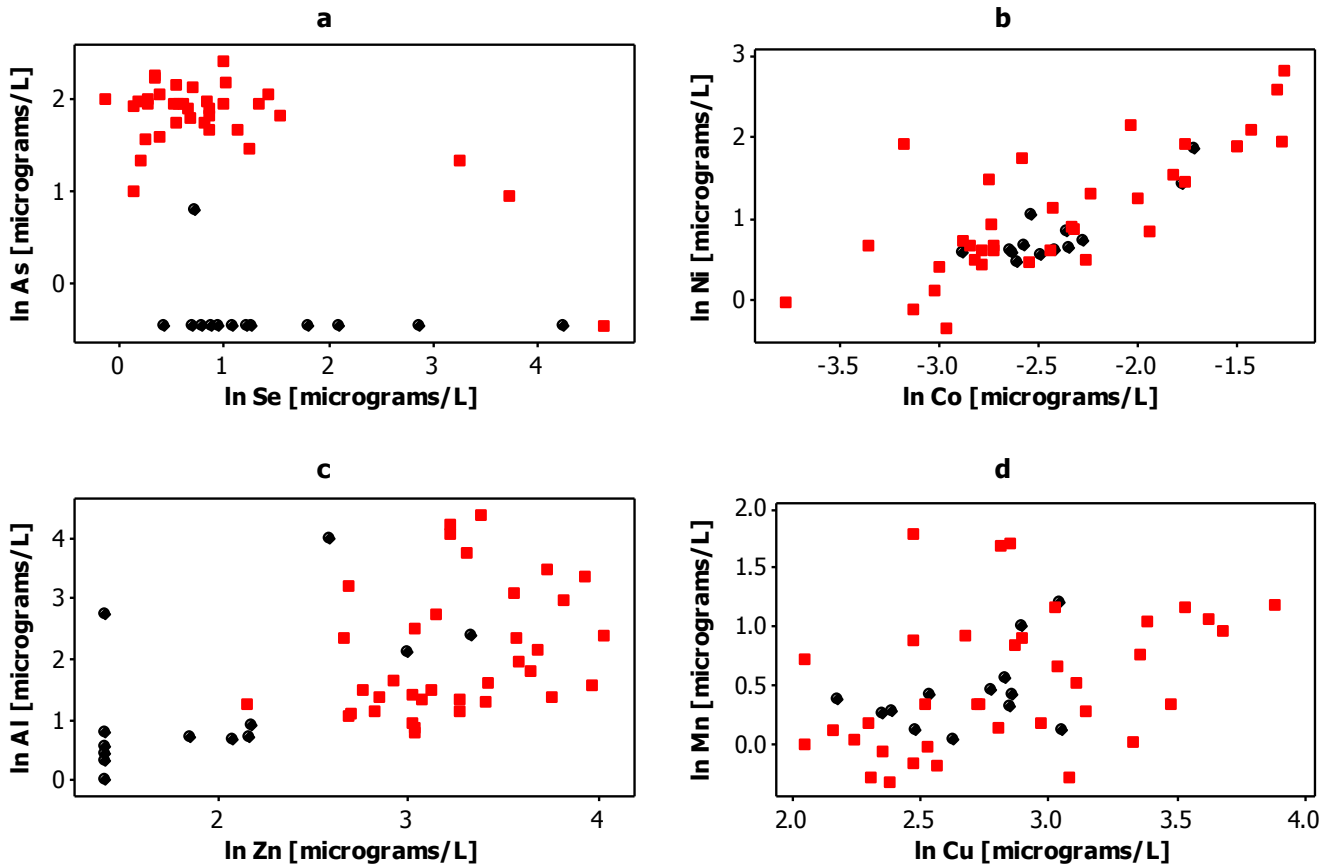


Figure 7-3: Correlation plots for some trace elements in CSF of MS patients (red squares) and CNT (black circles) from this study; a) arsenic *vs.* selenium, b) aluminium *vs.* zinc, c) manganese *vs.* cobalt, d) copper *vs* nickel.

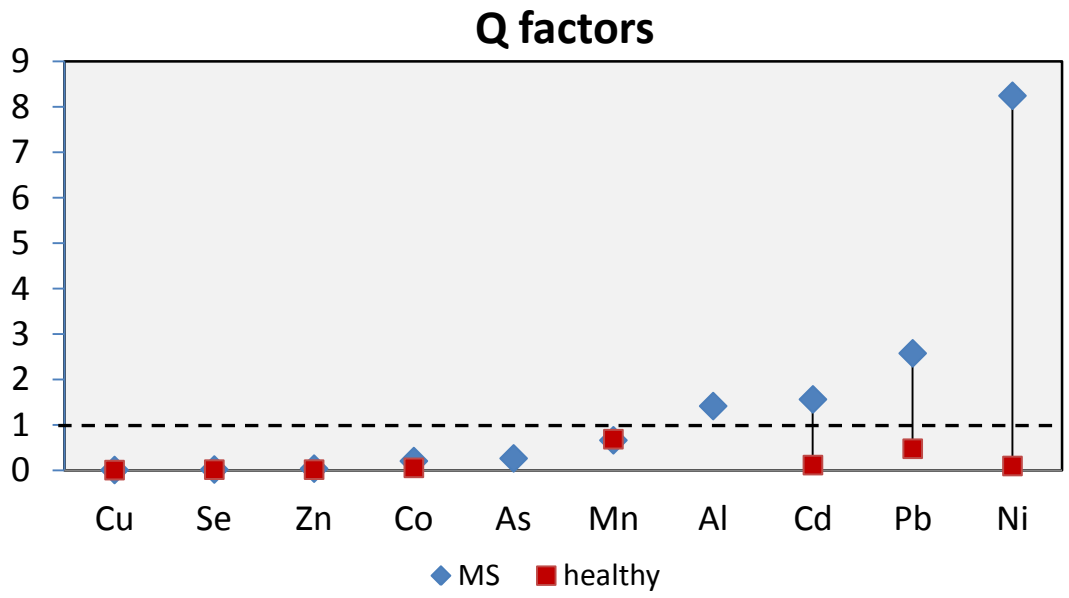
4.5 Q factors in MS differs from literature for toxic metals

For a subset of 7 MS patients, plasma trace element concentrations were measured and Q quotients calculated (element concentration in CSF/element concentration in plasma). Q quotients are used in the literature to investigate the leakage though the blood-CSF barrier (Gerhardsson *et al.,* 2008). A comparison between Q factors of MS from this study and reports in the literature available for healthy subjects and patients affected by Alzheimer Diseases (AD) and MS (Gellein *et al.* , 2008) is reported in Table 7-5.

Table 7-5: Q values (based on medians) for 7 MS patients from this study compared to: healthy and Alzheimer Diseases (AD) patients as reported by (1) (Gerhardsson et al., 2008) (median, with range given in parentheses); in the last column Q values have been calculated from mean reported values by (2) (Gellein et al., 2008) for patients affected by multiple sclerosis (MS).

Q	MS (n=7)	min	max	healthy (*n*=54) [1]	AD (*n*=264)[1]	MS [2]
QCu	0.01	0.01	0.03	0.01 (0.01-0.03)	0.01(0.01-0.11)	0.04
QSe	0.02	0.01	0.04	0.02 (0.00-0.04)	0.02 (0.00-0.07)	0.05
QZn	0.04	0.02	0.06	0.02 (0.01-0.20)	0.02 (0.00-0.19)	0.06
QCo	0.21	0.11	0.93	0.06(0.03-0.12)	0.09(0.02-0.76)	< 0.001
QAs	0.27	0.13	0.57	NA	NA	NA
QMn	0.67	0.45	1.77	0.69 (0.26-1.87)	0.45 (0.08-1.50)	0.45
QAl	1.42	0.43	3.68	NA	NA	NA
QCd	1.57	1.05	11.11	0.12 (0.00-1.07)	0.02 (0.00-4.04)	0.23
QPb	2.58	0.70	4.82	0.48 (0.00-2.55)	0.27 (0.00-2.40)	1.43
QNi	8.25	1.29	101.83	0.1 (0.01-0.03)	0.14 (0.01-0.11)	/

For essential trace elements (copper, selenium, zinc), there is a satisfactory agreement between Q values from MS patients from this study, and those reported for healthy subjects, AD patients (Gerhardsson *et al.,* 2008) and calculated for MS (Gellein *et al.,* 2008) (see Figure 7-4). Toxic elements such as Cd, Pb and Ni show a much higher Q value in MS from this study than healthy people, as shown in Figure 7-4. Note that for essential trace elements, such as Cu, Se, Zn, Co and Mn, values were highly comparable to those for healthy from the literature. However, for toxic metals, such as Cd, Pb and Ni, values were much higher in MS than in controls. Unfortunately, no data could be found for aluminium and arsenic.



Figures 7-4: Q factors (ratio of TE in CSF and plasma) calculated for 7 MS cases from this study (blue diamonds') and as reported for healthy volunteers (n=54) by (Gerhardsson et al., 2008) (red squares) based on medians.

4.6 Arsenic positively correlates to total proteins in CSF of MS

For a subset of 22 samples (9 MS and 13 CNT), correlation between total arsenic and total proteins in CSF could be explored. A Pearson test was undertaken to detect correlations between protein content and arsenic in the CSF. The analysis was carried out for for MS and CNT separately. Arsenic was not correlated to total protein content in the CNT group ($p = 0.154$) but was positively in the MS group ($p = 0.062$). The data and trends are reported in Table 7-6 and Figure 7-5.

Table 7-6: Correlation of arsenic and selenium and proteins in CSF

Factors	***n***	**Person correlation coefficient**	**P value**
As-proteins in MS	9	-0.419	0.154
As- proteins in CNT	13	-0.554	0.062
Se- proteins	22	-0.338	0.124

No significant correlation to proteins was found for other trace elements both in CSF of cases, controls or the overall group.

In contrast to what was observed for arsenic, selenium did not show a different pattern in CSF of MS and controls. There was a common negative weak relationship between

selenium and total proteins in both MS and CNT. However, this was not statistically significant.

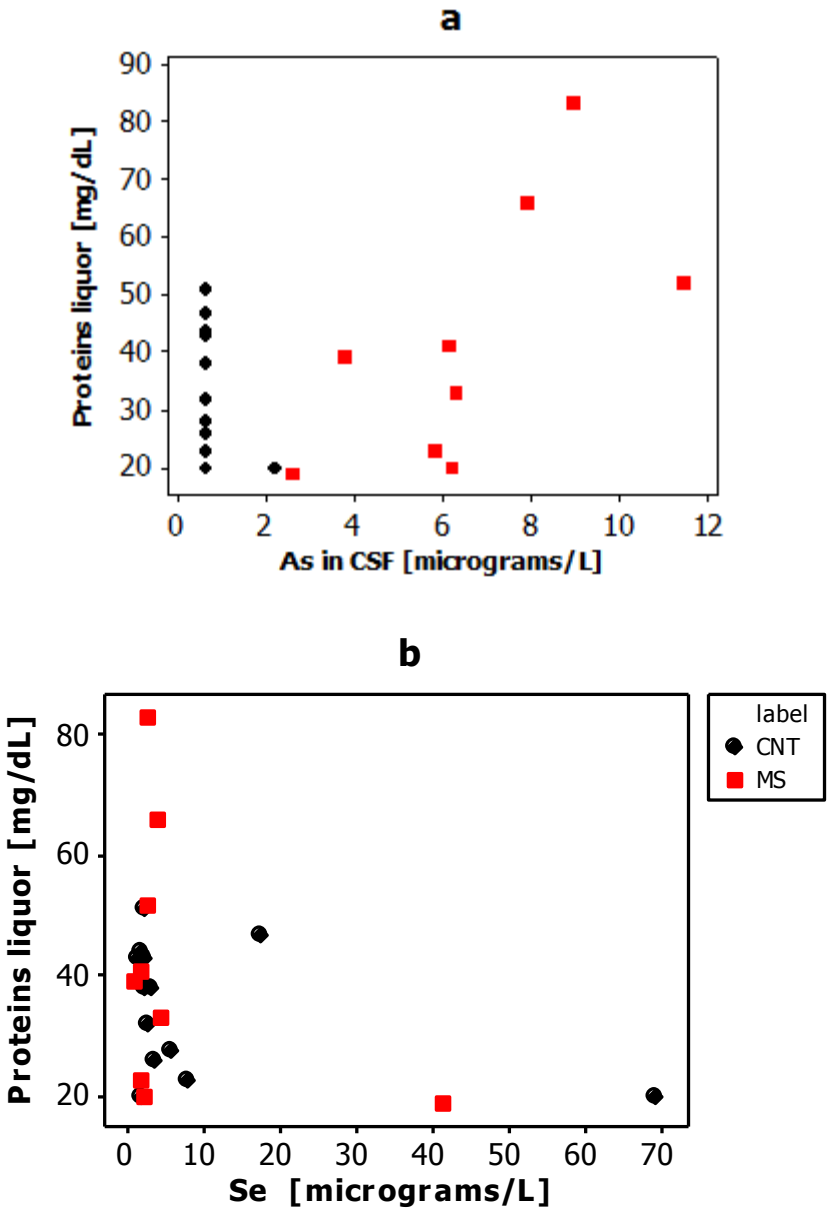


Fig. 7-5: a) Opposite correlation trends between arsenic and total proteins in CSF on controls (black circles) and multiple sclerosis patients in this study (red squares); b) on the contrary selenium and proteins show a similar negative correlation in CSF of both MS and CNT.

4.5 Comparing to existing literature on CSF

Few studies have reported arsenic concentration in CSF and there is a general need for producing and sharing data in order to understand what is "normal" and what is

"abnormal" in populations affected by neurological disorders. A comparison to existing literature for healthy, MS and other neurological condition patients is reported in Table 7-7 A and B.

Zn and Cu content in CSF for MS patients were comparable to levels reported previously (Gellein *et al.,* 2008; Melo *et al.,* 2003) (Figure 7-6 A). Melo *et al*. reported no significant difference in zinc in CSF of 18 MS cases compared to 19 controls. However, differences between Zn in CSF of MS and CNT in this chapter are greater than those reported by Melo *et al.* (2003) and Gellein *et al*. (2008). This difference resulted in a strong positive contribution to the two group's separation in OPLS-DA. This analysis reveals Zn as second only to arsenic in being the most important trace element for discriminating between MS and control patients. A Japanese population (both controls and Alzheimer Disease) reported by Hozumi *et al.* (2011), showed the lowest levels of zinc in CSF compared to other populations.

Copper levels found in both MS and CNT in this chapter (Figure 7-6 A) did not show the great variation identified for Zn, and did not become a significant feature in driving division between MS and CNT. These results agree with those reported by Gellein *et al.* (2008), but are in disagreement to those reported by Melo *et al.* (2003). Melo *et al.* (2003) show an increase in copper between MS and controls (MS mean: 10.90 SEM: 1.11 μg/L compared to CNT 9.10 SEM: 0.62 μg/L).

Manganese found in CSF of MS cases in this study was slightly higher than the values reported for patients affected by MS in Norway (Figure 7-7 A). However, the decrease in levels of Mn in MS reported by (Milo *et al.,* 2003) was not found. Cadmium and aluminium showed a particularly high concentration in the CSF of MS from this study compared both to CNT and to other reported levels in the CSF (see Figure 7.7 –B and C). Cadmium was higher in CSF of MS in this study group than CNT and MS cases from Norway (Gellein *et al.,* 2008).

Nickel levels in CSF of both cases and controls were quite high compared to levels reported in 1992 for the Italian population (Sabbioni *et al.*, 1992) (<0.25 μg/L) for 30 Italian subjects. However, a more recent study (Alimonti *et al*., 2007a) reports, for patients affected by Parkinson disease, values compatible to levels found in the study population presented in this thesis.

Selenium in CSF of MS of both controls and cases was higher than Se concentration in CSF of other reported populations from Italy and Norway (Gellein *et al*., 2008). This is consistent with significant levels of selenium detected in urine samples of volunteers from the city of Linguaglossa (in the proximity of Catania) compared to literature seen in Chapter 6.

In relation to arsenic, levels found in CSF of MS were higher than CNT and higher than those reported in Italian references by (Sabbioni, *et al.* 1992).

Table 7-7-A: Comparison of trace elements in CSF of this study to the values reported in the literature [µg/L]

	n	Al		Mn		Co		Ni		Cu	
Population		**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
CNT (Sicily) -TS	***13***	8.1	5.1	1.6	0.3	0.10	0.02	2.49	0.89	15.1	2.5
MS (Sicily)	***36***	14.7	3.2	2.04	0.23	0.11	0.01	4.05	0.59	19.0	1.6
CNT (Germany) -1	***29***	NA	NA	1.00	0.09	NA	NA	NA	NA	21.7	1.6
PD (Italy) - 2	***26***	2.3	0.3	0.63	0.08	NA	NA	NA	NA	23.7	3.2
CNT (Italy)- 2	***13***	2.9	0.1	0.85	0.10	NA	NA	NA	NA	22.5	2.2
MS (Norway) - 3	***9***	NA	NA	1.19	0.23	< LOD	NA	NA	NA	21.6	2.3
CNT(Norway)- 3	***13***	NA	NA	1.32	0.14	< LOD	NA	NA	NA	21.7	2.0
CNT (Japan) - 4	***15***	NA	NA	1.90	0.26	NA	NA	NA	NA	10.2	0.5
AD (Japan) - 4	***21***	NA	NA	1.80	0.20	NA	NA	NA	NA	17.4	2.3
MS (Norway) - 7	***18***	NA	NA	1.07	0.13	NA	NA	NA	NA	10.9	1.11
CNT (Norway) - 7	***19***	NA	NA	1.78	0.26	NA	NA	NA	NA	8.67	0.49
PD (Italy) - 5	***42***	2.15	0.16	0.69	0.06	0.09	0.01	3.34	0.56	19.4	1.2
CNT (Italy) - 5	***20***	2.64	0.11	0.95	0.09	0.13	0.01	5.40	0.74	21.9	1.1
CNT (Italy) - 6	***<10***	NA	NA	NA	NA	0.81	0.09	<0.25	NA	36.7	5.7

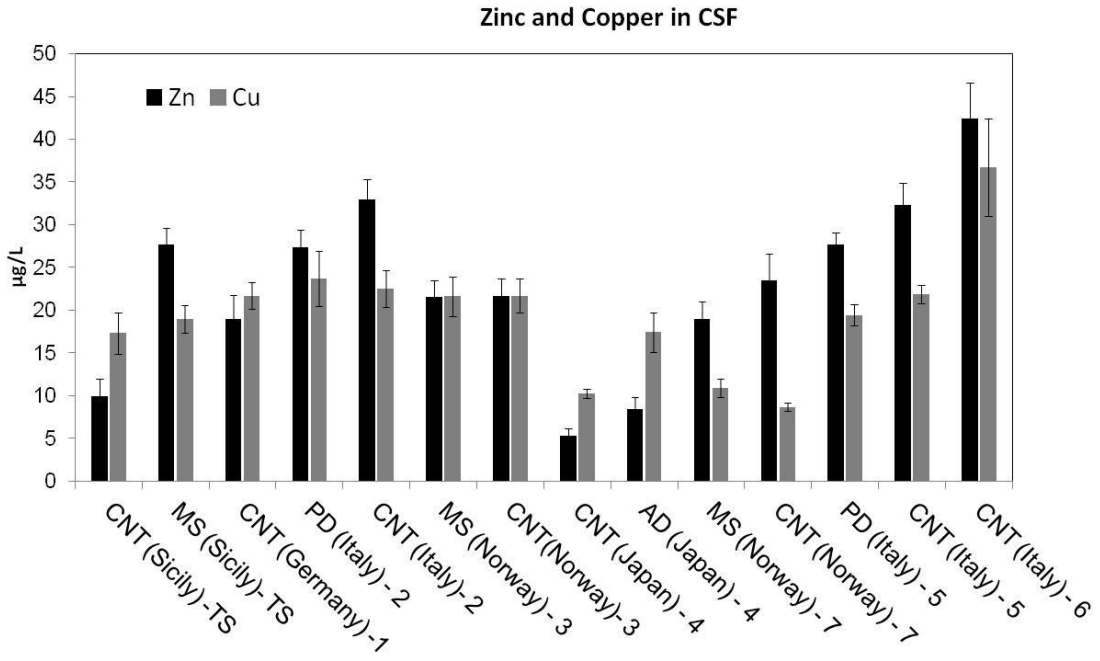
References: (TS) this study, (1) (Nischwitz et al., 2008), (2) (Forte et al., 2004), (3) (Gellein et al., 2008), (4) (Hozumi et al., 2011), (5) (Alimonti et al., 2007a), (6) (Sabbioni *et al.*, 1992), (7) (Melo et al., 2003). SEM = standard error, NA= not analysed.

Table 7-7-B: Comparison of trace elements in CSF of this study to the values reported in the literature [µg/L]

	n	Zn		Cd		Pb		As		Se		Mo	
Population		**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean***	**SEM***	**Mean**	**SEM**
CNT (Sicily) -TS	***13***	9.0	3.8	0.02	0.06	0.19	0.09	<LOD		9.5	5.1	0.36	0.10
MS (Sicily) -TS	***36***	27.7	2.0	0.08	0.04	0.44	0.06	6.5	0.4	6.6	3	0.55	0.1
CNT (Germany) -1	***29***	19	2.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PD (Italy) - 2	***26***	27.3	2.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
CNT (Italy)- 2	***13***	32.9	2.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
MS (Norway) - 3	***9***	21.5	2	0.040	0.010	0.76	0.13	NA	NA	2.28	0.3	0.22	0.04
CNT(Norway)- 3	***13***	21.7	2	0.040	0.007	0.59	0.07	NA	NA	2.5	0.21	0.42	0.1
CNT (Japan) - 4	***15***	5.3	0.9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
AD (Japan) - 4	***21***	8.4	1.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
MS (Norway) - 7	***18***	19.0	2.0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
CNT (Norway) - 7	***19***	23.5	3.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PD (Italy) - 5	***42***	27.7	1.4	0.04	0.003	0.46	0.04	NA	NA	NA	NA	0.33	0.03
CNT (Italy) - 5	***20***	32.3	2.5	0.05	0.01	0.91	0.08	NA	NA	NA	NA	0.45	0.06
CNT (Italy) - 6	***<10***	42.4	4.2	1.7	0.25	NA	NA	0.21	0.05	1.7	0.38	3.3	0.63

References: (TS) this study, (1) (Nischwitz et al., 2008), (2) (Forte et al., 2004), (3) (Gellein et al., 2008), (4) (Hozumi et al., 2011), (5) (Alimonti et al., 2007a), (6) (Sabbioni *et al.*, 1992), (7) (Melo et al., 2003). SEM = standard error, NA= not analysed.

A)



B)

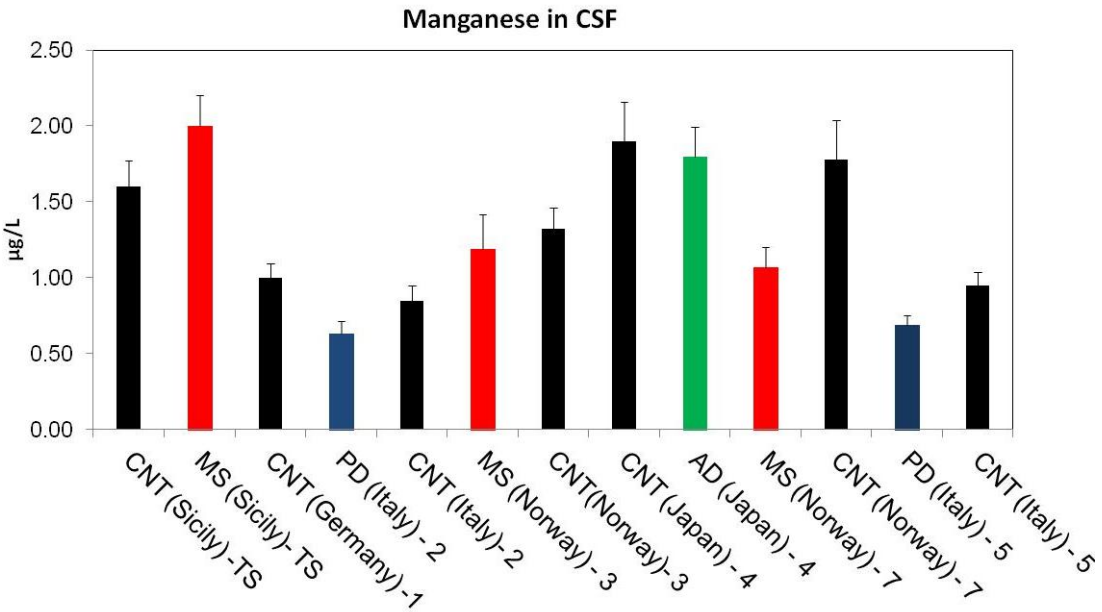


Figure 7-6: A) levels of zinc (black) and copper (grey) reported from this study and from literature for controls (CNT), multiple sclerosis (MS), Alzheimer's disease (AD) and Parkinson's disease patients (PD); country of origin of the study and volunteers is indicated in brackets; B) manganese values reported in CSF. In both cases graphs are based on means and error bars represent standard error. Data on Sicily are from this study. References: (1) (Nischwitz et al., 2008), (2) (Forte et al., 2004), (3) (Gellein et al., 2008), (4) (Hozumi et al., 2011), (5) (Alimonti et al., 2007a), (6) (Sabbioni et al., 1992), (7) (Melo et al., 2003).

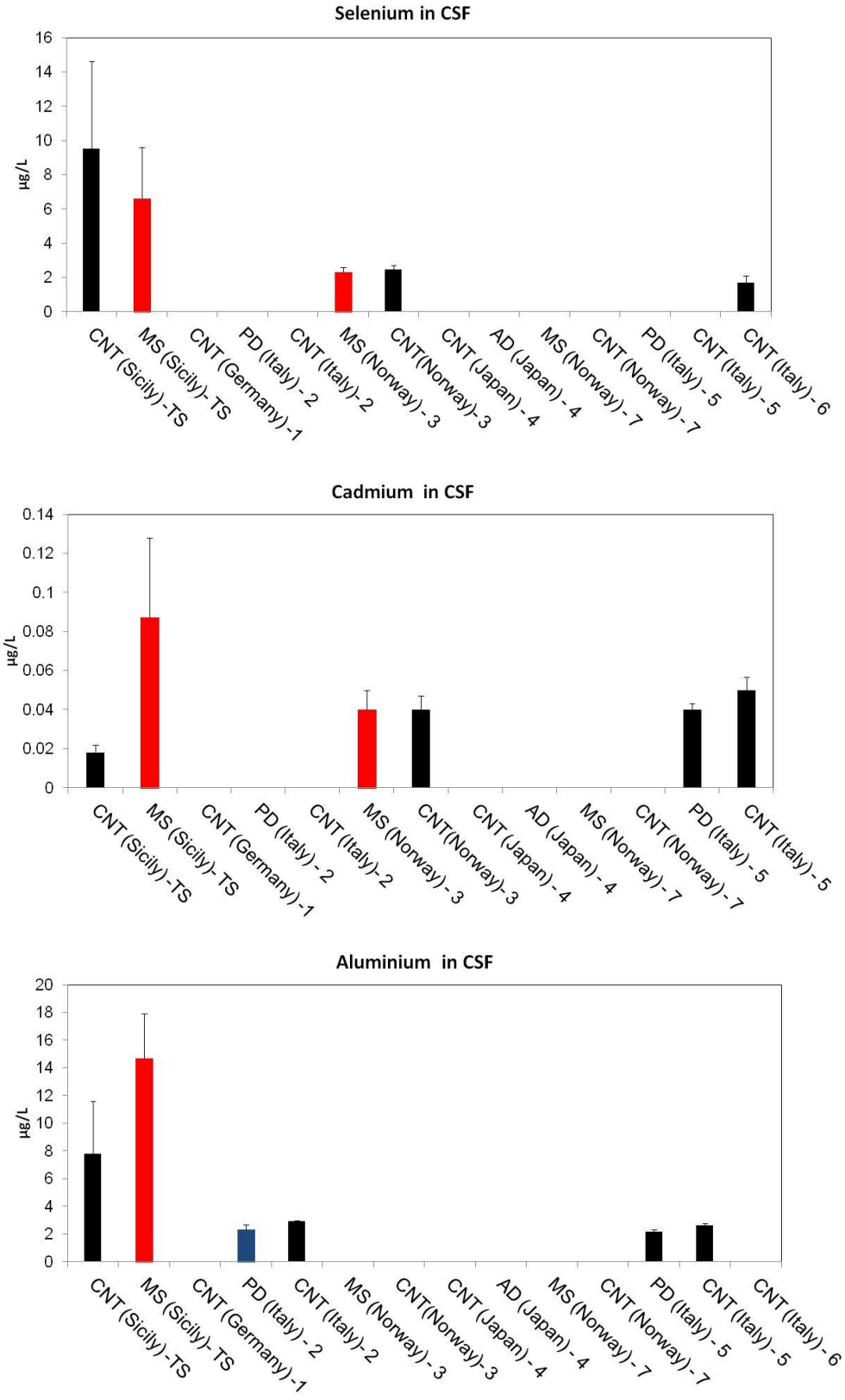


Figure 7-7: Levels of selenium (A) and cadmium (B) and aluminium (C) in study and from literature. References as in Figure 7-6.

5. Discussion

Studies on CSF are still quite rare mainly due to difficulties in accessing CSF samples. Unfortunately, the limited literature in the field appears to be contradictory with some authors reporting an imbalance of trace elements (Melo *et al*., 2003) in CSF of MS in comparison to controls whilst others report absence of significant differences (Gellein *et al*., 2008; Madeddu *et al*., 2011).

In this chapter, a significant difference in trace element composition of CSF of MS cases and CNT emerged. In fact, As, Zn, Pb, Al, Cd and Mo were significantly increased in CSF of MS than in CNT. Conversely, Se shows a trend of decrease in CSF of MS compared to controls. By means of a multivariate supervised approach, it was possible to separate MS and CNT CSFs. This confirmed the possibility of separating cases and controls by the analysis of CSF trace element composition in this study group. The main role in driving this separation is played by an increase of As, Zn and Pb in MS.

The increase in the levels of certain toxic metals and metalloids in the CSF of MS compared to controls from this study could be the result of an impairment of the blood brain barrier due to the MS and its progression. It is known that the blood–brain barrier is compromised in humans affected by MS and tight junction disruption is associated with serum protein leakage across the blood–brain barrier of MS (Plumb *et al*., 2002; Kirk *et al*., 2003). Along with proteins, toxic trace elements normally not present in the CSF because of sequestration by the choroid plexus might cross the barrier alone or bound to proteins and other molecules.

Arsenic levels positively correlated to CSF proteins in MS. Albumin is known for its exquisite ability to bind a wide array of molecules including metals and small organic compounds (Kragh-Hansen, 1990). Thus the correlation between increased As and protein levels in CSF could indeed prove the presence of a protein-carrier/ transporter (such as Human Serum Albumin), which transports small molecules and ions through the blood brain barrier in MS. In this chapter, a significant increase in zinc in the CSF of MS patients was found in respect to controls. However, Q Zn was not > 1 or different from the literature (Gerhardsson *et al.,* 2008). It is likely that the increase in Zn in MS patients reflects a global increase in serum levels of Zn in MS patients. If this was the case, these

data agree with findings by Ristori *et al.* (2011). This research group found a complex chemical element and oxidative status imbalance in the serum of patients with MS (Alimonti *et al.,* 2007b; Ristori *et al.* 2011). They found an increase in serum levels of Zn, (and Ca and Sn, which were not measured in this thesis study), and suggested the potential role of toxic exposure to Zn (Park *et al.*, 2002, Schiffer *et al.*, 2001b) or zinc involvement because component of metalloproteinase and metallothioneins, known to be involved in disease pathogenesis (Espejo and Martínez-Cáceres, 2005; Hartung 2000). Recently a study from Madeddu *et al*. (2011) examined the levels of trace elements in CSF of MS and controls from Sardinia. This is probably the most recent study in the field and they found no significant differences between the CSF of cases and controls apart from some differences at the 75th percentile for Zn with an increased level of this element being present in MS patients. This finding on zinc is consistent to what presented in the thesis. In this context, it is noteworthy that Schiffer *et al.* reported a MS cluster in the USA in the proximity of a Zn smelter (Schiffer *et al.*, 2001a). Further work on the role of Zn in MS needs probably to be carried out.

Manganese concentrations remained unchanged in MS and CNT from the present study. This is in line with the findings from Madeddu *et al.* (2011) but differs from Melo *et al.* (2003), who reported a decrease in MS compared to controls, and related this to alterations in glutamine synthetase, which contains Mn.

Lead and Cadmium were found to be higher in CSF of MS than in CNT in this chapter. This result disagrees with findings from Madeddu *et al*. (2011). Cadmium is able to inhibit the mitochondrial electron transfer chain and to induce superoxide radical formation (Wang *et al.,* 2004). A significant increase of Cd in CSF of MS was found in this Chapter. An outbreak of MS cases was reported in Ohio in association to high concentration of Cd in rivers and sewage (Wang *et al.,* 2004). Cigarette smoking by interacting with leukocyte antigen genotypes could also increase the likelihood of MS (Hedstrom *et al.,* 2011).

Ni, Cd, Pb and Al Q ratios (CSF/blood) were > 1, showing a higher concentration of such toxic elements in CSF than in blood of MS patients. However, aluminium levels in both MS and CNT are higher than the values reported in the literature (Forte *et al*., 2004; Alimonti *et al.,* 2007a). This result might need further confirmation on a larger cohort of patients and controls and possibly additional steps should be taken to ensure that there are no sources of possible sample contamination.

Data from this study show a negative correlation between selenium and proteins in both CSF of MS and CNT, the opposite of what is seen for As in MS. The difference in the behaviour of arsenic and selenium could be related to the nature of these elements. Selenium is an essential element that is required by the body in various biochemical processes and is associated with proteins that are important antioxidants. Speciation analysis of CSF, which has not yet been carried out, might reveal that Se in the CSF may be either inorganic elemental Se or Se complexed to very small organic molecules. These small species may be able to cross the blood brain barrier, but selenium ligated to large complex proteins will not. In contrast, the known As species are very small (iAs, DMA, MMA, AsB), and may readily cross the barrier, especially since they are not incorporated and utilised within the essential biochemical pathways.

It could be postulated that certain metals are elevated in the brain (through deposition over time). For instance, iron overload has been demonstrated in MS lesions (Singh and Zamboni, 2009). If this is the case, people with MS should take additional steps to reduce their exposure to toxic elements. This could be achieved through modification of their diet and life style. Besides occupational exposure in certain parts of the world, exposure to certain toxic elements such as arsenic are rather high due to presence of such elements in water, the food-chain and the atmosphere. Such types of exposure may further lead to adverse health outcomes in MS patients, including enhancing the progression of the disease. The cohort investigated here lives in close proximity to Mt. Etna, which, as explained in Chapters 2 and 6 is a major emitter of trace elements in air and groundwater. Furthermore, Sicilians are great consumers of fish, which is a vehicle of exposure for mercury, lead, cadmium (Damiano *et al.*, 2011) and some species of arsenic (Sirot *et al.*, 2009). Hence diet could represent an additive source of exposure to hazardous elements in both the general population and in MS patients.

6. Conclusions

This chapter demonstrated significant differences in the trace elemental profile of CSF of patients affected by MS in comparison to controls. Specific features requiring further attention are the increase in toxic metals and metalloids and the decrease in selenium in CSF of patients affected by MS. Speciation studies are recommended for the future in order to investigate chemical species, identify their role in different biological processes including their effects on the brain.

7. Acknowledgments

Dr. Jackie Morton of the Health and Safety Laboratory is acknowledged for collaborating on this project. Prof. Alessandra Nicoletti and her research team who contribute to this work by providing the samples. I would like to thank all the volunteers affected by Multiple Sclerosis and the controls from Linguaglossa.

APPENDIX

Appendix to Chapter 1

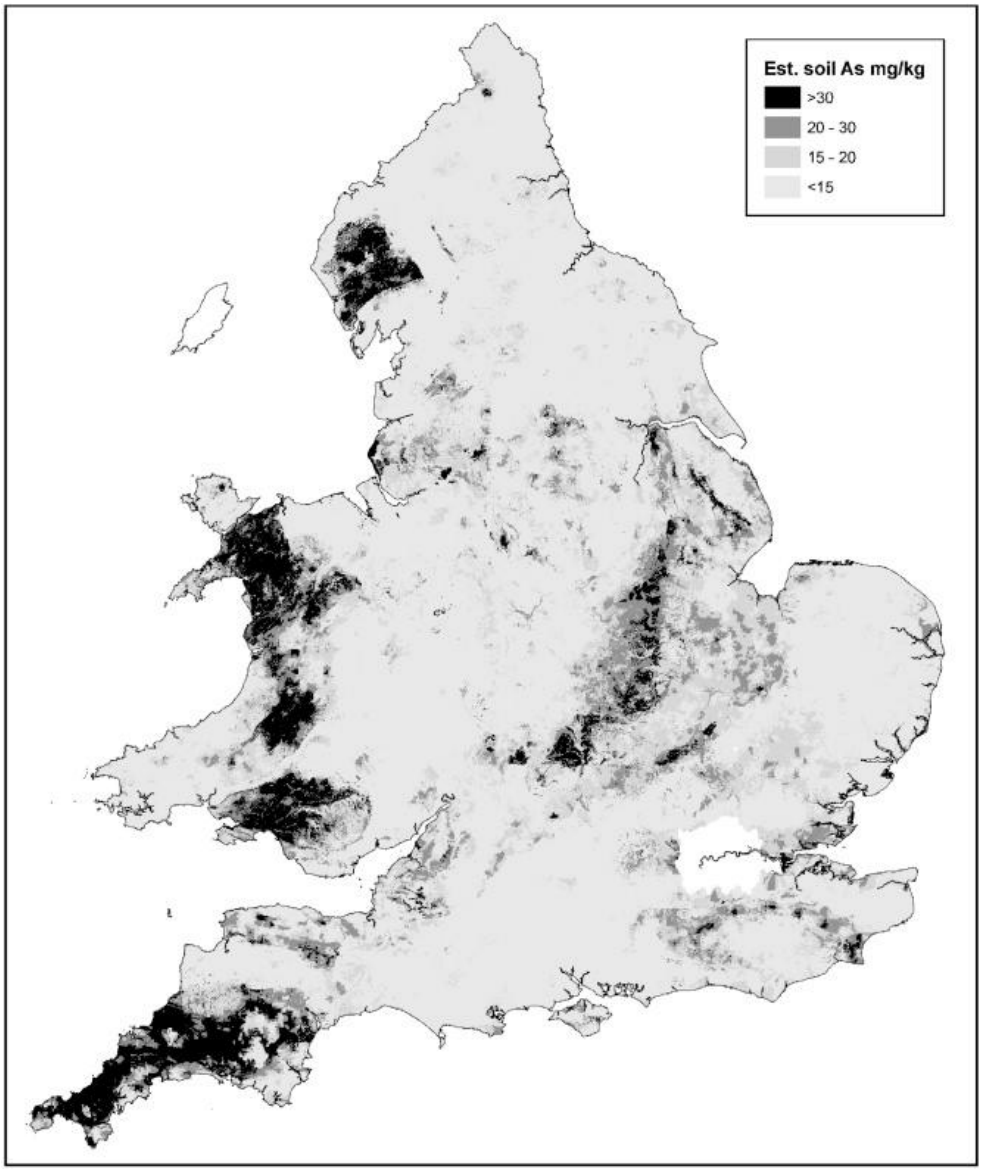


Figure A1-1: Categorical map of mineral topsoil equivalent geometric mean As (mg kg−1) for England and Wales from (Appleton et al., 2008).

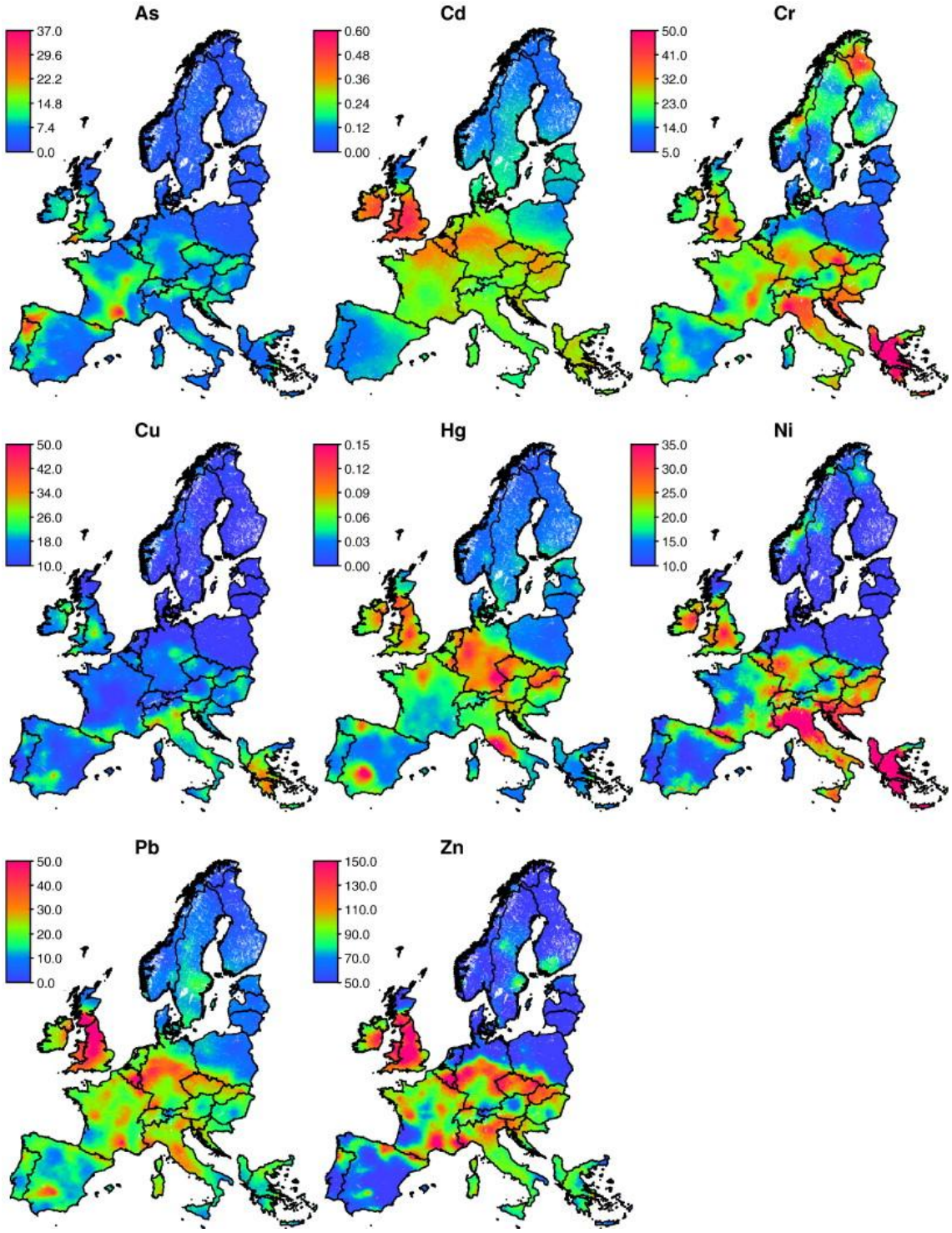


Figure A1-2 Maps of heavy metal concentrations in topsoil [mg kg^{-1}] interpolated using block regression-kriging from (Rodríguez Lado *et al.*, 2008).

Appendix of Chapter 3

Table A3-1: Total arsenic in urine of the 49 volunteers involved in this study: Bangladeshi (Bangl) and Caucasians (Cauc) without specific gravity correction (seven measurements)

	Sample name	Ethnicity	As_t [µg /L]
1	**NX 066**	Bangl	6.01 ± 1.48
2	**NX 002**	Bangl	66.1 ± 3.1
3	**NX 004**	Bangl	43.3 ± 5.5
4	**NX 005**	Bangl	41.4 ± 3.6
5	**NX 008**	Bangl	30.7 ±1.8
6	**NX 009**	Bangl	130 ± 5
7	**NX 011**	Bangl	61.7 ± 5.7
8	**NX 013**	Bangl	11.7 ± 2.3
9	**NX 017**	Bangl	151.2 ± 6.4
10	**NX 018**	Bangl	164 ± 7
11	**NX 019**	Bangl	19.7 ± 1.6
12	**NX 022**	Bangl	26.5 ± 2.6
13	**NX 025**	Bangl	56.2 ± 4.03
14	**NX 033A**	Bangl	25.6 ± 3.8
15	**NX 034**	Bangl	17.5 ± 2.8
16	**NX 042 B**	Bangl	26.6 ± 2.5
17	**NX 067**	Bangl	11.4 ± 2.0
18	**NX 081**	Bangl	44.1 ± 4.6
19	**O 31**	Bangl	21.3 ± 2.4
20	**O 19**	Bangl	4.54 ±1.52
21	**O 38**	Bangl	5.53 ± 0.99
22	**O 45**	Bangl	16.7 ± 2.6
23	**O 49**	Bangl	206 ± 10
24	**O10**	Bangl	8.64 ± 1.07
25	**O14**	Bangl	30.0 ± 3.0
26	**O18**	Bangl	22.4 ± 3.8
27	**O20**	Bangl	22.2 ± 1.6
28	**O22**	Bangl	22.7 ± 1.5
29	**O23**	Bangl	18.8 ± 2.9
30	**O24**	Bangl	13.5 ± 1.5
31	**O25**	Bangl	7.83 ± 0.52
32	**O30**	Bangl	25.9 ± 3.0
33	**O33**	Bangl	45.5 ± 2.0
34	**O46**	Bangl	73.0 ± 4.5
35	**O47**	Bangl	46.9 ± 2.9
36	**OO4**	Bangl	36.3 ±2.3
37	**OO7**	Bangl	41.8 ±1.7
38	**UK34**	Cauc	94.3 ± 8.8
39	**UK7**	Cauc	35.1 ± 8.1
40	**UK21**	Cauc	270 ± 6
41	**UK55**	Cauc	76.5 ± 4.57
42	**UK5**	Cauc	5.15 ± 1.01

	Sample name	Ethnicity	As_t [µg /L]
43	**UK9**	Cauc	14.5 ± 2.7
44	**UK30**	Cauc	15.4 ± 1.5
45	**UK22**	Cauc	285 ± 67
46	**UK44**	Cauc	29.4 ± 3.5
47	**UK8**	Cauc	9.51 ± 3.11
48	**UK60**	Cauc	73.4 ± 17.1
49	**LEMR112ND**	Cauc	13.7 ± 2.4

Table A3-2: Ratio of urinary CA/DMA for some reported groups

Country	Ethnicity	Population type	CA/DMA ratio	Reference
UK	*Bangladeshi*	general	0.23	This study
UK	*not specified*	timber workers	0.23	(Morton and Mason, 2006)
USA	*Mexican American*	general	0.25	(Caldwell et al., 2009)
USA	*mixed*	no sea food 24 h	0.25	(Navas-Acien et al., 2010)
USA	*Non-Hispanic white*	general	0.42	(Caldwell et al., 2009)
USA	*Non-Hispanic black*	general	0.54	(Caldwell et al., 2009)
Germany	*not specified*		0.54	(Heitland and Kolster, 2008)
UK	*Somali*	general	0.58	(Brima *et al.*, 2006b)
UK	*Caucasians*	general	1.03	(Brima *et al.*, 2006b)
Japan	*Japanese (males)*		1.44	(Hata et al., 2007)
USA	*mixed*	sea food 24 h	1.70	(Navas-Acien et al., 2010)
Japan	*NIES CRM*		1.88	(Hata et al., 2007)
UK	*not specified*	general	2.09	(Morton and Mason, 2006)
UK	*Asians*	general	3.52	(Brima *et al.*, 2006b)
UK	*Caucasians*	general fish 24h	**4.88**	This study
UK	*not specified*	general fish 24h	**5.39**	(Morton and Mason, 2006)

Appendix of Chapter 4

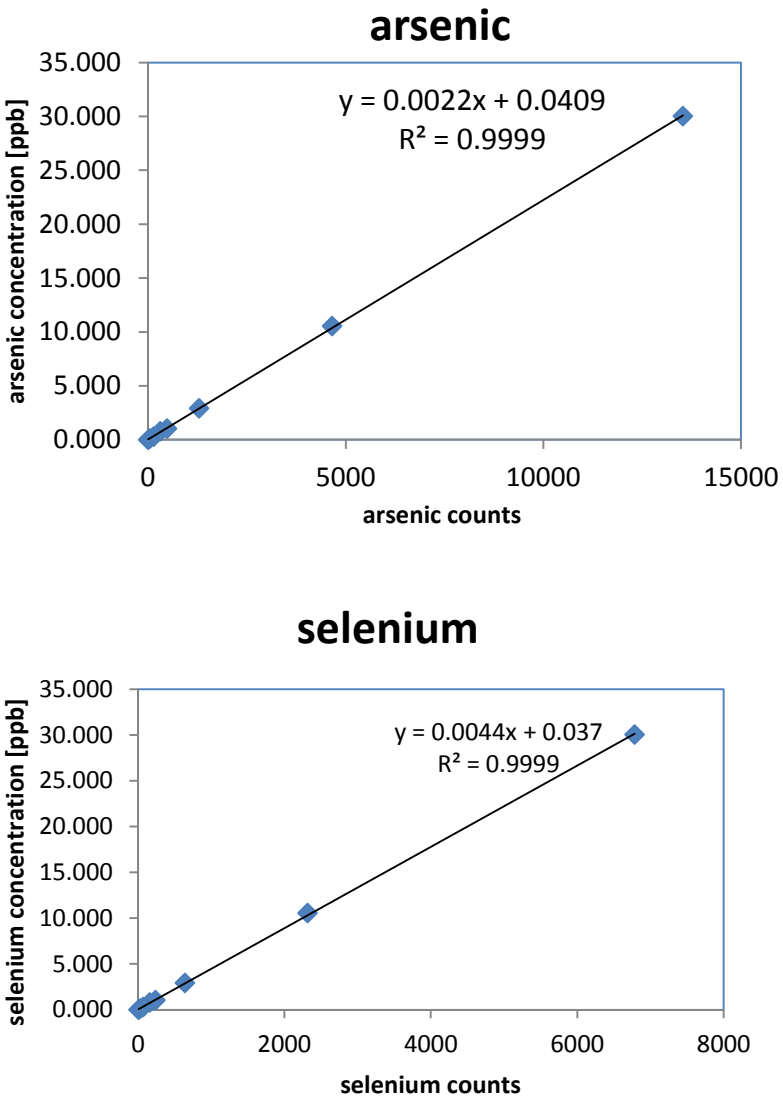


Figure A4-1: Example of calibration curves for arsenic and selenium, used to convert counts into concentration.

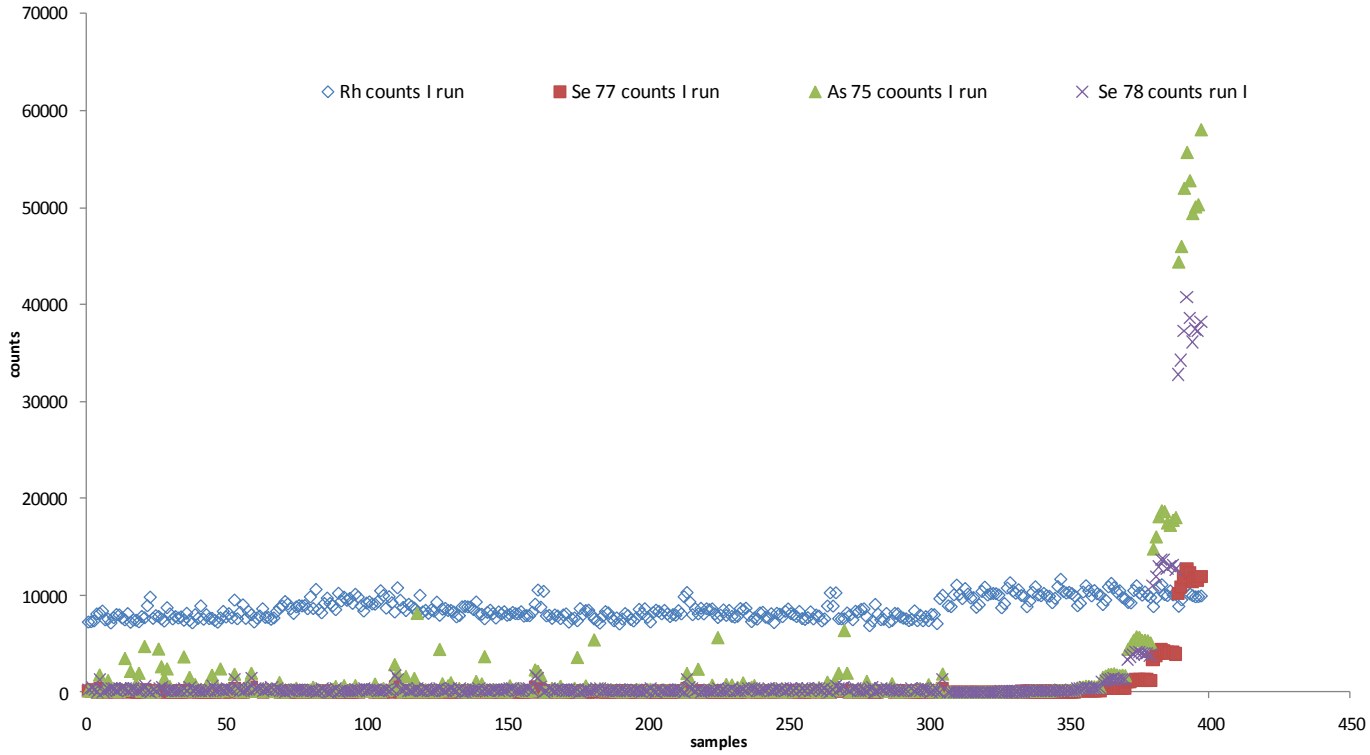


Figure A4-2: Counts for rhodium 103, selenium 77, arsenic 75 and selenium 78 for run I. Higher values are the external standards used for calibration.

Table A4-1:Counts (as average of 7 readings) for rhodium (isotope 103) and selenium (isotope 78) for external standards (std0-9) and urine samples. Standards were read every 40 measurements and calibration curves re-calculated. For selenium, concentrations are shown with (Se 78) and without Rh correction (Se 78 no Rh). The latter was used chosen.

	Counts		Concentrations	
	Rh103	**Se78**	**Se 78 corrected**	**Se 78 no Rh correction**
std0	46963.29	7.14	0.01	0.1
std1	47482.11	31.91	0.13	0.2
std2	47412.38	70.96	0.31	0.3
std3	47990.89	160.01	0.72	0.7
std4	48695.08	239.06	1.08	1.1
std5	48906.09	641.47	2.92	2.9
std6	49103.94	2316.47	10.55	10.2
std7	48631.84	6784.24	31.25	29.9
std8	47967.73	22417.55	104.74	98.7
std9	47266.98	67644.03	320.79	297.7
1	47681.07	20.95	0.07	0.1
2	32859.21	898.64	6.11	4.0
3	35400.78	922.45	5.82	4.1
4	34555.86	2404.12	15.57	10.6
5	33888.3	891.97	5.88	4.0
6	37594.09	325.25	1.92	1.5
7	37495.02	364.78	2.16	1.6
8	35195.35	717.67	4.55	3.2
9	32301.74	671.47	4.64	3.0
10	34774.41	1073.42	6.90	4.8
11	38319.37	251.44	1.45	1.1
12	32844.7	874.84	5.95	3.9
13	32583.38	1052.94	7.22	4.7
14	33057.77	706.71	4.77	3.1
15	38414.72	1674.93	9.75	7.4
16	36487.31	1077.23	6.59	4.8
17	35252.95	841.97	5.33	3.7
18	34074.37	1074.37	7.04	4.8
19	32167.98	490.98	3.40	2.2
20	37288.83	1042.94	6.25	4.6
21	30909.09	635.75	4.59	2.8
22	33836.63	580.51	3.82	2.6
23	34388.5	689.09	4.47	3.1
24	35259.14	267.16	1.67	1.2
25	32824.89	596.23	4.05	2.7
26	33469.27	651	4.34	2.9
27	33285.52	438.12	2.93	2.0
28	29760.01	524.8	3.93	2.3
29	32162.38	365.73	2.53	1.6
30	35608.73	80	0.48	0.4
31	41169.59	2301.71	12.51	10.2
32	35809.45	354.78	2.20	1.6
33	32002.89	845.78	5.90	3.8

	Counts		Concentrations	
	Rh103	**Se78**	**Se 78 corrected**	**Se 78 no Rh correction**
34	31349.22	293.35	2.07	1.3
35	29535.28	275.73	2.07	1.3
36	30424.25	732.91	5.38	3.3
37	29969.65	664.33	4.95	3.0
38	32385.01	434.31	2.98	1.9
39	29046.28	345.26	2.64	1.6
40	29288.44	622.9	4.74	2.8
std0	37199.48	4.76	0.01	0.1
std1	38625.55	22.38	0.10	0.1
std2	38894.34	61.43	0.32	0.3
std3	39143.83	134.29	0.71	0.7
std4	39497.91	191.44	1.01	1.0
std5	39556.36	573.37	3.05	3.0
std6	39881.32	1986.41	10.52	10.4
std7	39400.13	5782.88	31.04	30.1
std8	40387	19981.25	104.66	103.9
std9	38575.35	58337.39	319.95	303.4

Table A4-2:Demographics and trace elements in urine of 161 volunteers monitored in the UK group (divided by ethnic groups) values are in µg/L. SG is specific gravity.

Bangladeshis

ICP	sample id	AGE	GENDER	SG	Se	Se SG	As	As SG	Cu	Cu SG	Zn	Zn SG
407	O 45	31	F	1.014	9.6	11.0	12.9	14.8	27.4	31.3	213	243
411	NX 033A	38	M	1.013	11.5	14.2	19.0	23.4	29.6	36.5	140	173
426	NX 012	36	F	1.006	6.1	16.3	22.4	59.8	14.2	37.8	167	446
427	O46	72	M	1.017	19.7	18.5	52.9	49.8	31.1	29.3	797	750
428	O 19	33	M	1.004	3.1	12.6	3.0	12.2	7.2	28.9	187	750
429	O 39	52	M	1.025	32.2	20.6	19.5	12.5	27.8	17.8	566	362
430	O 49		M	1.02	26.6	21.3	152.4	121.9	25.5	20.4	2125	1700
435	O14	44	M	1.018	16.8	14.9	21.4	19.0	35.1	31.2	192	170
436	NX 013	93	F	1.004	1.9	7.7	9.2	37.0	14.8	59.1	132	530
451	NX 0 66	37	M	1.004	3.4	13.5	4.1	16.5	15.8	63.3	37	147
452	NX O42		F	1.004	3.7	14.6	4.7	18.8	25.6	102.5	31	123
457	NX048		M	1.017	35.1	33.1	18.8	17.7	22.1	20.8	632	595
458	NX 022	28	M	1.011	17.2	25.0	20.2	29.3	27.5	39.9	294	427
459	NX 019	51	F	1.01	7.2	11.5	14.8	23.7	17.4	27.8	501	802
464	NX 049			1.005	6.4	20.6	4.5	14.4	8.4	27	48	152
470	NX 018	28	F	1.023	30.1	21.0	120.9	84.1	36.7	25.5	469	326
471	NX 017	55	M	1.023	33.6	23.4	118.8	82.6	40.3	28	593	412
472	NX 002	47	M	1.019	26.7	22.5	49.0	41.3	29.9	25.1	436	367
473	NX 041	30	F	1.01	15.1	24.2	31.5	50.4	33.4	53.4	535	856
476	NX 015	65	M	1.015	16.2	17.3	35.1	37.4	65.2	69.5	736	785
477	NX 081	45	M	1.022	25.0	18.2	31.6	23.0	28.6	20.8	595	433
478	NX069	47	M	1.008	5.7	11.5	6.9	13.7	12.7	25.4	175	349
479	NX 008	56	M	1.01	12.4	19.8	23.2	37.2	71	113.6	356	569
480	NX 010	51	M	1.015	13.1	14.0	22.2	23.6	22.3	23.8	537	573
482	NX 004	29	M	1.017	18.4	17.3	31.9	30.1	34.2	32.1	360	339
483	NX 088			1.021	22.6	17.2	12.8	9.7	39.5	30.1	261	199
485	NX 025	48	F	1.008	9.4	18.9	45.1	90.1	14.6	29.3	100	200
487	NX 087			1.015	15.2	16.2	14.4	15.3	175.7	187.4	253	270
488	NX 034	33	F	1.009	10.9	19.5	13.4	23.9	25.4	45.2	334	593
489	NX 036	52	M	1.014	19.4	22.1	85.9	98.2	22.8	26	240	274
490	NX 009	71	F	1.013	21.5	26.4	112.3	138.2	199.5	245.6	538	662
491	NX 029	40	M	1.012	19.0	25.3	24.1	32.1	16.3	21.7	692	923
492	O25	60	M	1.005	3.2	10.3	5.4	17.3	24.3	77.9	164	525
493	O23	59	M	1.017	15.8	14.9	13.3	12.5	35.6	33.5	115	108
494	NX 005	36	F	1.014	17.7	20.2	31.0	35.4	57.1	65.3	607	694
495	NX 011	34	M	1.02	28.6	22.8	45.7	36.6	24.5	19.6	1064	851
498	OO7	67	M	1.014	15.6	17.8	32.4	37.1	31.6	36.1	201	229
503	NX 067	52	M	1.01	7.7	12.3	7.8	12.5	21.2	33.9	593	948
506	NX 023	30	M	1.006	5.7	15.1	38.9	103.7	23.5	62.5	176	470
507	OO4		F	1.016	26.4	26.4	29.0	29.0	29.3	29.3	915	915
508	O10	68	F	1.006	4.7	12.5	6.7	18.0	10.8	28.9	257	685
509	NX 045 B			1.023	34.0	23.7	26.2	18.2	37.2	25.9	479	333
512	NX 057	39	M	1.009	4.3	7.6	2.2	3.8	20.8	37	83	148

ICP	sample id	AGE	GENDER	SG	Se	Se SG	As	As SG	Cu	Cu SG	Zn	Zn SG
513	O30	42	M	1.01	12.5	20.0	19.9	31.8	16.1	25.7	109	175
516	O22	38	M	1.021	22.4	17.1	17.0	12.9	27.3	20.8	549	418
517	O18	29	M	1.012	6.7	8.9	16.8	22.4	26.9	35.8	157	210
518	NX 045 A			1.018	25.4	22.6	20.2	18.0	19.7	17.5	557	495
519	O20	45	M	1.014	13.3	15.2	16.6	19.0	22.9	26.2	234	267
523	O47	72	F	1.019	19.9	16.8	38.1	32.1	20.1	16.9	328	276
533	O 38	49	M	1.008	6.1	12.2	3.8	7.6	51.6	103.2	220	440
535	A 039	54	F	1.018	34.0	30.2	11.2	10.0	30.6	27.2	1653	1469
538	O24	67	M	1.012	7.3	9.8	10.3	13.8	23.4	31.2	290	387
539	NX 027	50	M	1.025	36.1	23.1	78.5	50.2	48.7	31.2	986	631
541	NX 065	47	M	1.007	10.5	23.9	11.2	25.6	13.3	30.3	195	445

Indians

ICP	sample id	AGE	GENDER	SG	Se	Se SG	As	As SG	Cu	Cu SG	Zn	Zn SG
72	22 m	69	M	1.015	14.6	15.5	11.18	11.9	13.9	14.8	210	224
84	uk 28	37	F	1.029	26.0	14.4	10.52	5.8	26.8	14.8	797	439
87	uk 10	38	F	1.033	33.5	16.2	7.43	3.6	32.9	16	788	382
89	uk 11	38	M	1.012	20.8	27.8	11.97	16.0	18.1	24.2	579	772
143	8081024	21	M	1.028	26.0	14.8	78.14	44.7	20.2	11.5	574	328
158	09081019a	23	M	1.023	5.3	3.7	102.94	71.6	25.9	18	817	568
182	rk11		M	1.028	5.3	3.0	3.98	2.3	35	20	621	355
186	09081001a		M	1.029	3.5	1.9	2.65	1.5	11.9	6.6	733	404
193	8081013		M	1.024	2.6	1.7	2.12	1.4	15.3	10.2	289	193
196	09081011a	23	M	1.027	3.4	2.0	1.58	0.9	10.3	6.1	576	341
206	8081021		M	1.023	28.8	20.1	31.76	22.1	29.2	20.3	387	269
208	8081012		M	1.024	25.7	17.1	5.53	3.7	12.2	8.1	567	378
210	09081002a	26	M	1.02	21.7	17.3	2.18	1.7	12.5	10	393	314
211	8081004	26	M	1.027	23.0	13.6	8.24	4.9	34	20.2	603	358
212	8081015		M	1.026	21.7	13.4	17.79	10.9	39.2	24.1	858	528
214	8081023		M	1.032	21.5	10.8	33.60	16.8	15.1	7.5	533	267
227	8081010	21	M	1.024	15.6	10.4	17.89	11.9	30.9	20.6	598	398
234	09081003a		M	1.026	22.4	13.8	7.18	4.4	15.1	9.3	956	588
239	8081003		M	1.018	4.9	4.3	2.16	1.9	7.8	7	151	134
247	8081027	37	M	1.033	18.6	9.0	14.06	6.8	18.2	8.8	611	296
252	09081012a	21	M	1.024	19.4	12.9	7.28	4.9	16.7	11.1	462	308
257	11f	20	F	1.027	36.8	21.8	19.95	11.8	19	11.2	369	218
263	uk 11	38	M	1.013	11.5	14.2	9.68	11.9	13.1	16.1	500	616
268	8081012		M	1.022	22.6	16.5	6.74	4.9	31.9	23.2	367	267
276	8081002			1.009	14.6	25.9	6.18	11.0	16	28.5	330	587

Pakistani

ICP	sample id	AGE	GENDER	SG	Se	Se SG	As	As SG	Cu	Cu SG	Zn	Zn SG
140	09081013a		M	1.031	24.0	12.4	25.80	13.3	16.9	8.7	689	356
148	8081019		M	1.021	11.9	9.0	22.86	17.4	5.6	4.3	340	259
149	8081011	21	M	1.028	25.2	14.4	58.41	33.4	17.1	9.8	340	194
154	8081007	21	M	1.027	7.0	4.1	20.33	12.0	28.2	16.7	636	377
157	2f	22	F	1.018	4.2	3.8	18.46	16.4	17.6	15.6	415	369
159	8081009	21	M	1.009	1.0	1.8	0.50	0.9	16.8	29.8	198	352
160	8f	18	M	1.018	2.3	2.1	0.50	0.4	49.5	44	626	556
165	3f	50	F	1.015	3.7	3.9	0.50	0.5	198	211.2	1584	1689
175	10f	49	F	1.01	1.0	1.6	0.25	0.4	1.9	3	138	221
178	8081006	21	M	1.035	2.6	1.2	0.50	0.2	9.9	4.5	645	295
183	15f	18	F	1.016	2.1	2.1	0.50	0.5	6.8	6.8	573	573
187	09081009a	21	M	1.016	2.7	2.7	0.89	0.9	16.3	16.3	735	735
197	9f	19	F	1.026	6.1	3.7	0.50	0.3	40	24.6	417	256
198	7f	22	F	1.024	5.8	3.8	2.21	1.5	29	19.3	1035	690
209	8081018	27	M	1.026	27.1	16.7	52.62	32.4	78.3	48.2	854	525
213	09081017a	21	M	1.027	17.3	10.2	9.44	5.6	10.5	6.2	159	94
229	09081007a	19	M	1.021	17.9	13.6	5.38	4.1	13.6	10.4	676	515
237	09081012a	21	M	1.031	21.4	11.0	28.26	14.6	14.9	7.7	742	383
249	12 m	20	M	1.014	12.1	13.8	5.01	5.7	26.6	30.4	405	463
266	8081008	19	M	1.026	40.8	25.1	542.20	333.7	11.6	7.2	323	199
278	8081025	23	M	1.033	54.2	26.3	61.96	30.0	44.7	21.7	2265	1098

Caucasians

ICP	sample id	AGE	GENDER	SG	Se	Se SG	As	As SG	Cu	Cu SG	Zn	Zn SG
74	uk 8	22	F	1.014	21.6	24.7	8.52	9.7	15.6	17.9	295	337
76	uk 14			1.015	15.5	16.5	12.92	13.8	11.5	12.2	368	393
79	uk 34	21	F	1.022	35.1	25.5	81.16	59.0	18.5	13.5	422	307
80	uk 16		M	1.016	23.1	23.1	14.26	14.3	13.5	13.5	1207	1207
86	uk 17	25	F	1.018	24.3	21.6	102.66	91.2	19.2	17.1	348	309
88	uk 22	36	F	1.022	33.7	24.5	233.83	170.1	17.2	12.5	102	74
91	uk 44	26	M	1.018	28.0	24.9	24.69	21.9	31.7	28.2	520	462
101	uk9	24	F	1.026	47.9	29.5	11.21	6.9	25.7	15.8	642	395
120	uk 12	39	F	1.013	16.2	19.9	19.42	23.9	9.4	11.6	291	358
125	le mr 11			1.02	3.3	2.6	1.71	1.4	11.7	9.4	283	226
161	uk52	33	M	1.029	4.8	2.7	2.82	1.6	16.4	9	570	314
162	uk30	31	M	1.023	3.1	2.2	1.58	1.1	4.7	3.2	216	150
169	uk42	28	M	1.016	3.5	3.5	7.92	7.9	5.4	5.4	535	535
170	uk40	22	F	1.018	6.5	5.8	11.75	10.4	10.3	9.1	528	469
ICP	**SAMPLE**	**AGE**	**GENDER**	**SG**	**Se**	**Se SG**	**As**	**As SG**	**Cu**	**Cu SG**	**Zn**	**Zn SG**

191	uk60	28	M	1.022	5.7	4.2	7.75	5.6	19.9	14.5	481	350
222	dav leic	29	M	1.026	33.1	20.4	16.87	10.4	12.4	7.7	523	322
240	cla leic no fish	28	F	1.011	13.0	18.9	7.71	11.2	4.4	6.3	274	399
250	uk32	30	M	1.024	20.6	13.7	9.90	6.6	9.9	6.6	585	390
260	uk 36	41	F	1.008	4.4	8.7	2.51	5.0	8.5	17	240	479
262	13 f	31	F	1.012	17.3	23.1	12.81	17.1	14.3	19.1	130	173
264	uk 55	49	F	1.018	11.0	9.7	65.37	58.1	1.6	1.4	74	65
270	uk 36	29	M	1.027	20.5	12.1	27.98	16.6	7.1	4.2	261	155
279	mr 12 2nd	28	M	1.029	48.7	26.9	74.22	41.0	19.1	10.5	742	410

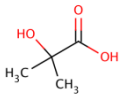
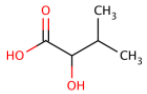
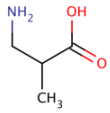
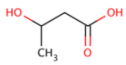
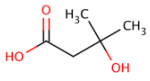
Mixed

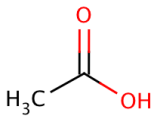
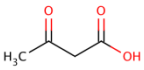
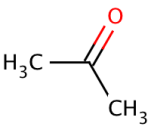
ICP	sample id	AGE	GENDER	SG	Se	Se SG	As	As SG	Cu	Cu SG	Zn
69	uk 21	60	F	1.011	12.5	18.3	256.40	372.9	17.1	24.9	241
70	uk 61			1.019	22.4	18.8	56.99	48.0	19.1	16.1	181
71	17 f	41	F	1.02	30.0	24.0	7.88	6.3	35.8	28.7	592
73	18 m	33	M	1.019	22.0	18.5	8.58	7.2	17.1	14.4	602
75	uk 2	33	M	1.028	49.6	28.3	382.39	218.5	23.4	13.4	627
81	uk 3	30	M	1.013	19.1	23.5	28.38	34.9	13.2	16.2	126
82	19 m	41	M	1.017	17.4	16.3	6.69	6.3	17.6	16.6	524
83	uk13			1.018	17.0	15.1	61.53	54.7	11	9.7	115
90	uk 45			1.03	41.6	22.2	19.79	10.6	31.8	17	468
109	uk 04			1.016	26.3	26.3	95.41	95.4	6.7	6.7	575
156	16m	24	F	1.013	2.4	2.9	3.50	4.3	16.7	20.6	282
164	14m	45	M	1.015	2.7	2.9	2.50	2.7	7.4	7.9	183
167	20f	25	F	1.019	1.0	0.8	11.12	9.4	4.3	3.6	498
168	uk64			1.016	2.1	2.1	1.28	1.3	4.9	4.9	348
174	uk 31	41	M	1.024	1.0	0.7	0.76	0.5	12.6	8.4	74
179	uk1	29	M	1.016	3.6	3.6	13.45	13.5	5.2	5.9	301
189	sr 12	26	F	1.016	3.6	3.6	3.17	3.2	13.1	13.1	194
192	6f	22	F	1.03	5.9	3.1	3.06	1.6	14.9	8	2551
204	4f	20	F	1.023	1.0	0.7	0.50	0.3	25.4	17.6	656
218	le 304008			1.022	22.2	16.1	53.67	39.0	22.4	16.3	1199
219	le a 1108			1.013	13.0	16.0	15.43	19.0	8.8	10.9	171
221	le a 12 08			1.03	30.1	16.0	471.43	251.4	19.1	10.2	498
225	le a 10 08			1.015	22.4	23.9	10.51	11.2	5.6	6	547
228	09081004a		M	1.024	21.2	14.1	12.91	8.6	14.9	10	246
235	09081018a	21	M	1.02	11.8	9.5	13.66	10.9	17.4	13.9	442
238	rk 23/03/09 no fish	25	F	1.022	14.2	10.3	20.94	15.2	5.4	3.9	300

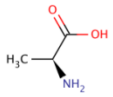
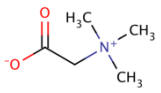
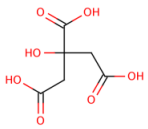
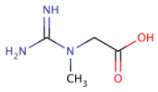
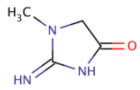
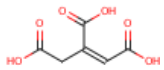
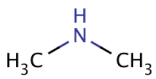
251	090810124b		M	1.018	22.4	19.9	18.14	16.1	10.2	9.1	506
ICP	**sample id**	**AGE**	**GENDER**	**SG**	**Se**	**Se SG**	**As**	**As SG**	**Cu**	**Cu SG**	**Zn**
253	1m	21	F	1.017	20.7	19.5	24.10	22.7	11	10.4	431
259	uk 51		M	1.019	29.2	24.6	219.18	184.6	13.4	11.3	1003
261	uk 62	41	M	1.018	14.8	13.2	15.06	13.4	23.8	21.2	355
265	uk 66			1.02	19.9	15.9	6.93	5.5	10.4	8.3	333
269	uk 54	28	M	1.022	12.9	9.4	74.72	54.3	22.9	16.7	550
271	5f	21	F	1.027	42.5	25.2	65.23	38.7	47.3	28	644
273	uk 38	30	M	1.016	14.9	14.9	43.51	43.5	8.8	8.8	163
274	uk 26	33	M	1.009	10.4	18.5	18.43	32.8	6	10.7	133
277	uk33			1.012	44.7	59.7	35.29	47.1	24.6	32.8	460
437	O 31			1.009	14.4	25.6	16.3	28.9	14.3	25.5	125
443	MORNING ROO4			1.019	23.9	20.1	33.8	28.5	22.9	19.3	400

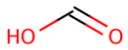
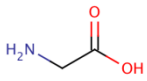
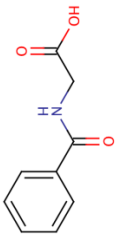
Appendix to Chapter 5

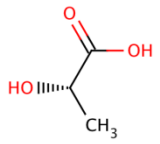
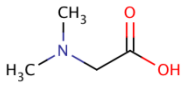
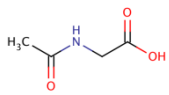
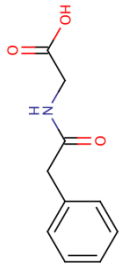
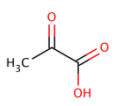
Table A5-1: Urinary metabolites monitored in this study (source www.hmdb.ca)

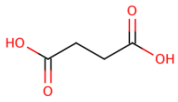
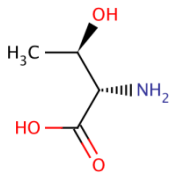
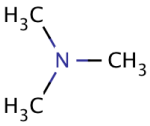
Metabolite	Formula	Main biological Function
2-Hydroxyisobutyrate		Alpha-Hydroxyisobutyric acid is a metabolite of methyl tert-butyl ether (MTBE). MTBE may be obtained through environmental exposure. MTBE is rapidly eliminated from the body, mainly through expired air as the unchanged compound. MTBE is to some extent metabolised to t-butyl alcohol (TBA) and formaldehyde and oxidised to 2-methyl-1,2-propanediol and a-hydroxy isobuturic acid. Alpha-Hydroxyisobutyric acid has been used as an arial bactericide.
2-Hydroxyisovalerate		2-Hydroxy-3-methylbutyric acid is a metabolite found in the urine of patients with Phenylketonuria, Methylmalonic acidemia, Propionic acidemia, 3-Ketothiolase deficiency, Isovaleric acidemia, 3-Methylcrotonylglycemia, 3-Hydroxy-3-methylglutaric acidemia, Multiple carboxylase deficiency, Glutaric aciduria, Ornithine transcarbamylase deficiency, glyceroluria, Tyrosinemia type 1, Galactosemia, and Maple syrup urine disease.
3-Aminoisobutyrate		beta-aminoisobutyric acid is the product from the conversion of N-carbamyl-beta-aminoisobutyric acid by the enzyme Beta-ureidopropionase, the last step in pyrimidine degradation. Beta-ureidopropionase deficiency is an inborn error of pyrimidine degradation associated with neurological abnormalities.
3-Hydroxybutyrate		3-Hydroxybutyric acid is a ketone body. Like the other ketone bodies levels of 3-hydroxybutyrate in blood and urine are raised in ketosis. In humans, 3-hydroxybutyrate is synthesized in the liver from acetyl-CoA, and can be used as an energy source by the brain when blood glucose is low. Blood levels of 3-hydroxybutyric acid levels may be monitored in diabetic patients to look for diabetic ketoacidosis. Persistent mild hyperketonemia is a common finding in newborns. Ketone bodies serve as an indispensable source of energy for extrahepatic tissues, especially the brain and lung of developing mammals. Another important function of ketone bodies is to provide acetoacetyl-CoA and acetyl-CoA for synthesis of cholesterol, fatty acids, and complex lipids. During the early postnatal period, acetoacetate and beta-hydroxybutyrate are preferred over glucose as substrates for synthesis of phospholipids and sphingolipids in accord with requirements for brain growth and myelination. Thus, during the first 2 weeks of postnatal development, when the accumulation of cholesterol and phospholipids accelerates, the proportion of ketone bodies incorporated into these lipids increases. On the other hand, an increased proportion of ketone bodies is utilized for cerebroside synthesis during the period of active myelination. In the lung, AcAc serves better than glucose as a precursor for the synthesis of lung phospholipids.
3-Hydroxyisovalerate		3-Hydroxyisovaleric acid is a normal human metabolite excreted in the urine. Elevated levels of this compound are found in several inherited disorders such as Dihydrolipoamide dehydrogenase Deficiency, 3-Methylcrotonyl-CoA carboxylase 1 deficiency and 3-Hydroxy-3-methylglutaryl-CoA lyase deficiency. 3-Hydroxyisovaleric acid is also elevated in smokers, in subjects undergoing long-term anticonvulsant therapy with carbamazepine and/or phenytoin. These levels are elevated due to impairment of renal reclamation of biotin. Levels may also be increased from prolonged consumption of raw egg-whites.

Metabolite	Formula	Main biological Function
Acetate		Acetic acid is one of the simplest carboxylic acids. The acetyl group, derived from acetic acid, is fundamental to the biochemistry of virtually all forms of life. When bound to coenzyme A it is central to the metabolism of carbohydrates and fats. However, the concentration of free acetic acid in cells is kept at a low level to avoid disrupting the control of the pH of the cell contents. Acetic acid is produced and excreted by certain bacteria, notably the *Acetobacter genus* and *Clostridium acetobutylicum*.
Acetoacetate		It is a weak organic acid and can be produced in the human liver under certain conditions of poor metabolism leading to excessive fatty acid breakdown (diabetes mellitus leading to diabetic ketoacidosis), it is then partially converted to acetone by decarboxylation and excreted either in urine or through respiration. Persistent mild hyperketonemia is a common finding in newborns. These compounds serve as an indispensable source of energy for extrahepatic tissues, especially the brain and lung of developing rats. Another important function of ketone bodies is to provide acetoacetyl-CoA and acetyl-CoA for synthesis of cholesterol, fatty acids, and complex lipids. During the early postnatal period, acetoacetate and beta-hydroxybutyrate are preferred over glucose as substrates for synthesis of phospholipids and sphingolipids in accord with requirements for brain growth and myelination. During the first 2 wk of postnatal development, when the accumulation of cholesterol and phospholipids accelerates, the proportion of ketone bodies incorporated into these lipids increases. On the other hand, an increased proportion of ketone bodies are utilized for cerebroside synthesis during the period of active myelination. In the lung, AcAc serves better than glucose as a precursor for the synthesis of lung phospholipids. The acid is also present in the metabolism of those undergoing starvation or prolonged physical exertion as part of gluconeogenesis. When ketone bodies are measured by way of urine concentration, acetoacetic acid, along with beta-hydroxybutyric acid or acetone, is what is detected.
Acetone		Is a ketone body produced during ketoacidosis. Acetone is not regarded as a waste product of metabolism. Its physiological role in biochemical machinery is not clear. A model for the role of acetone metabolism is presented that orders the events occurring in acetonemia in sequence: in diabetic ketosis or starvation, ketone body production (b-hydroxy-butyrate, acetoacetate) provides fuel for vital organs (such as heart and brain) raising the chance of survival of the metabolic catastrophe. However, when ketone body production exceeds the degrading capacity, the accumulating acetoacetic acid presents a new challenge to the pH regulatory system. Acetone production and its further degradation to C3 fragments fulfill two purposes: the maintenance of pH buffering capacity and provision of fuel for peripheral tissues. Since ketosis develops under serious metabolic circumstances, all the mechanisms that balance or moderate the effects of ketosis enhance the chance for survival. From this point of view, the theory that transportable C3 fragments can serve as additional nutrients is a novel view of acetone metabolism which introduces a new approach to the study of acetone degradation, especially in understanding its physiological function and the interrelationship between liver and peripheral tissues. Acetone is typically derived from acetoacetate through the action of microbial acetoacetate decarboxylases found in gut microflora. In chemistry, acetone is the simplest representative of the ketones.

Metabolite	Formula	Main biological Function
Alanine		Alanine is a nonessential amino acid. It is highly concentrated in muscle and functions as a major energy source. It is important participant and regulator in glucose metabolism and an important amino acid for lymphocyte reproduction and immunity.
Betaine		Betaine is a methyl donor that carries and donates methyl functional groups for the regeneration of S-adenosylmethionine (in the process consuming homocysteine). Tissue betaine is the major store of metabolically available methyl groups in mammals. It is important to proper liver function, cellular replication, and detoxification reactions. It is a major osmolyte, responsible for cell volume regulation in many tissues and counters urea cytotoxicity in kidneys.
Citrate		Citric acid (citrate) is formed in the tricarboxylic acid cycle or that may be introduced with diet. Urinary citrate excretion is used differential diagnosis of kidney stones and renal tubular acidosis. The secretory epithelial cells of the prostate gland of humans have a unique citrate-related metabolic pathway regulated by testosterone and prolactin and secrete extraordinarily high levels of citrate.
Creatine		Creatine is an amino acid that occurs in vertebrate tissues and in urine. In muscle tissue, creatine generally occurs as phosphocreatine. Creatine is excreted as creatinine in the urine. Creatine functions as part of the cell's energy shuttle. The high energy phosphate group of ATP is transferred to creatine to form phosphocreatine in the following reaction: Cr + ATP <-> PCr + ADP. This reaction is reversibly catalyzed by creatine kinase. In the human body creatine is synthesized mainly in the liver by the use of parts from three different amino acids - arginine, glycine, and methionine. 95% of it is later stored in the skeletal muscles, with the rest in the brain, heart, testes
Creatinine		Creatinine is a breakdown product of creatine phosphate in muscle. The loss of water molecule from creatine results in the formation of creatinine. Creatinine is transferred to the kidneys by blood plasma, where upon it is eliminated from the body by glomerular filtration and partial tubular excretion. Creatinine is usually produced at a fairly constant rate by the body. Measuring serum creatinine is a simple test and it is the most commonly used indicator of renal function. A rise in blood creatinine levels is observed only with marked damage to functioning nephrons; therefore this test is not suitable for detecting early kidney disease. Creatine and creatinine are metabolized in the kidneys, muscle, liver and pancreas.
***cis*-Aconitate**		An intermediate in the tricarboxylic acid cycle produced by the dehydration of citric acid. The enzyme aconitase (aconitate hydratase) catalyses the stereo-specific isomerization of citrate to isocitrate via cis-aconitate in the tricarboxylic acid cycle.
Dimethylamine		Dimethylamine is an organic secondary amine. It is a colorless, liquefied and flammable gas with an ammonia and fish-like odor. Dimethylamine is abundantly present in human urine. Main sources of urinary dimethylamine have been reported to include TMAO, a common food component, and asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide (NO) synthesis. ADMA is excreted in the urine in part unmetabolized and in part after hydrolysis to DMA by dimethylarginine dimethylaminohydrolase (DDAH). Significant increases in urinary DMA have been found in individuals after the consumption of fish and seafoods. The highest values were obtained for individuals that consumed coley, squid and whiting with cod, haddock, sardine, skate and swordfish.

Metabolite	Formula	Main biological Function
Formate		Formic acid is the simplest carboxylic acid. Formate is an intermediate in normal metabolism. It takes part in the metabolism of one-carbon compounds and its carbon may appear in methyl groups undergoing transmethylation. It is eventually oxidized to carbon dioxide. Formate is typically produced as a byproduct in the production of acetate. It is responsible for both metabolic acidosis and disrupting mitochondrial electron transport and energy production by inhibiting cytochrome oxidase activity, the terminal electron acceptor of the electron transport chain. Cell death from cytochrome oxidase inhibition by formate is believed to result partly from depletion of ATP, reducing energy concentrations so that essential cell functions cannot be maintained. Furthermore, inhibition of cytochrome oxidase by formate may also cause cell death by increased production of cytotoxic reactive oxygen species (ROS) secondary to the blockade of the electron transport chain. In nature, formic acid is found in the stings and bites of many insects of the order Hymenoptera, including bees and ants. The principal use of formic acid is as a preservative and antibacterial agent in livestock feed. When sprayed on fresh hay or other silage, it arrests certain decay processes and causes the feed to retain its nutritive value longer.
Glycine		Glycine is a simple, nonessential amino acid, although experimental animals show reduced growth on low-glycine diets. The average adult ingests 3 - 5 grams of glycine daily. Glycine is involved in the body's production of DNA, phospholipids and collagen, and in release of energy. Glycine levels are effectively measured in plasma in both normal patients and those with inborn errors of glycine metabolism. Nonketotic hyperglycinaemia is an autosomal recessive condition caused by deficient enzyme activity of the glycine cleavage enzyme system. The glycine cleavage enzyme system comprises four proteins: P-, T-, H- and L-proteins. The glycine cleavage system catalyses the oxidative conversion of glycine into carbon dioxide and ammonia, with the remaining one-carbon unit transferred to folate as methylenetetrahydrofolate. It is the main catabolic pathway for glycine and it also contributes to one-carbon metabolism. Patients with a deficiency of this enzyme system have increased glycine in plasma, urine and cerebrospinal fluid (CSF) with an increased CSF: plasma glycine ratio.
Hippurate		Hippuric acid is an acyl glycine formed by the conjugation of benzoic aicd with glycine. Acyl glycines are produced through the action of glycine N-acyltransferase which is an enzyme that catalyzes the chemical reaction: acyl-CoA + glycine < -- > CoA + N-acylglycine. Hippuric acid is a normal component of urine and is typically increased with increased consumption of phenolic compounds (tea, wine, fruit juices). These phenols are converted to benzoic acid which is then converted to hippuric acid and excreted in the urine. Hippuric acid is the most frequently used biomarker in the biological monitoring of occupational exposure to toluene. The concentration of hippuric acid in the urine of individuals exposed to a low toluene concentration does not differ from that of individuals not exposed to the solvent. This has led to the conclusion that hippuric acid should not be utilized in the biological monitoring of occupational exposure to low levels of toluene in the air. Protein-bound organic acids such as hippuric acid are markedly accumulated in uremic plasma and produce defective protein binding of drugs.

Metabolite	Formula	Main biological Function
Lactate		Lactic acid plays a role in several biochemical processes and is produced in the muscles during intense activity. Lactate measurement in the critically ill has been traditionally used to stratify patients with poor outcome. However, plasma lactate levels are the result of a finely tuned interplay of factors that affect the balance between its production and its clearance. When the oxygen supply does not match its consumption, organisms such as man who are forced to produce ATP for their integrity adapt in many different ways up to the point when energy failure occurs. Lactate, being part of the adaptive response, may then be used to assess the severity of the supply/demand imbalance. In such a scenario, the time to intervention becomes relevant: early and effective treatment may allow the cell to revert to a normal state, as long as the oxygen machinery is intact. Conversely, once the mitochondria are deranged, energy failure occurs even in the presence of normoxia. The lactate increase in critically ill patients may therefore be viewed as an early marker of a potentially reversible state. A number of studies have demonstrated that malignant transformation is associated with an increase in glycolytic flux and in anaerobic and aerobic cellular lactate excretion. Lactate dehydrogenase was found to be upregulated in most of these tumors compared to surrounding normal tissue.
***N,N*-Dimethylglycine**		Dimethylglycine (DMG) is an amino acid derivative found in the cells of all plants and animals and can be obtained in the diet in small amounts from grains and meat. The human body produces DMG when metabolizing choline into Glycine. Dimethylglycine that is not metabolized in the liver is transported by the circulatory system to body tissue. DMG is also a byproduct of homocysteine metabolism. Homocysteine and betaine are converted to methionine and N, N-dimethylglycine by betaine-homocysteine methyltransferase.
***N*-Acetylglycine**		Excessive amounts N-acetyl amino acids including N-acetylglycine (as well as N-acetylserine, N-acetylglutamine, N-acetylglutamate, N-acetylalanine, Nacetylmethionine and smaller amounts of N-acetylthreonine, N-acetylleucine, N-acetylvaline and N-acetylisoleucine) can be detected in the urine with individuals with Acylase I defiency. This enzyme is involved in the degradation of N-acylated proteins. Individuals with this disorder will experience convulsions, hearing loss ond difficulty feeding.
***N*-Phenylacetylglycine**		Phenylacetylglycine is an acyl glycine. Acyl glycines are normally minor metabolites of fatty acids. However, the excretion of certain acyl glycines is increased in several inborn errors of metabolism. In certain cases the measurement of these metabolites in body fluids can be used to diagnose disorders associated with mitochondrial fatty acid beta-oxidation. Acyl glycines are produced through the action of glycine N-acyltransferase which is an enzyme that catalyzes the chemical reaction: acyl-CoA + glycine < -- > CoA + N-acylglycine Phenylacetylglycine or PAG is a glycine conjugate of phenylacetic acid. Phenylacetic acid may arise from exposure to styrene (plastic) or through the consumption of fruits and vegetables. PAG is a putative biomarker of phospholipidosis. Urinary PAG is elevated in animals exhibiting abnormal phospholipid accumulation in many tissues and may thus be useful as a surrogate biomarker for phospholipidosis.
Pyruvate		An intermediate compound in the metabolism of carbohydrates, proteins, and fats. In thiamine deficiency, its oxidation is retarded and it accumulates in the tissues, especially in nervous structures. It is an intermediate in primary metabolism including fermentation processes. Present in muscle in redox equilibrium with Lactic acid.

Metabolite	Formula	Main biological Function
Succinate		Succinic acid is a dicarboxylic acid. The anion, succinate, is a component of the citric acid cycle capable of donating electrons to the electron transfer chain. Succinate dehydrogenase (SDH) plays an important role in the mitochondria, being both part of the respiratory chain and the Krebs cycle. SDH with a covalently attached FAD prosthetic group, binds enzyme substrates (succinate and fumarate) and physiological regulators (oxaloacetate and ATP). Oxidizing succinate links SDH to the fast-cycling Krebs cycle portion where it participates in the breakdown of acetyl-CoA throughout the whole Krebs cycle. The succinate can readily be imported into the mitochondrial matrix by the n-butylmalonate- (or phenylsuccinate-) sensitive dicarboxylate carrier in exchange with inorganic phosphate or another organic acid, e. g. malate. Mutations in the four genes encoding the subunits of the mitochondrial respiratory chain succinate dehydrogenase are associated with a wide spectrum of clinical presentations (i.e.: Huntington's disease).
Threonine		Threonine is an essential amino acid in humans. It is abundant in human plasma, particularly in newborns. Severe deficiency of threonine causes neurological dysfunction and lameness in experimental animals. Threonine is an immunostimulant which promotes the growth of thymus gland. It also can probably promote cell immune defense function. This amino acid has been useful in the treatment of genetic spasticity disorders and multiple sclerosis. It is highly concentrated in meat products, cottage cheese and wheat germ. The threonine content of most of the infant formulas currently on the market is approximately 20% higher than the threonine concentration in human milk. Due to this high threonine content the plasma threonine concentrations are up to twice as high in premature infants fed these formulas than in infants fed human milk. Threonine catabolism in mammals appears to be due primarily (70-80%) to the activity of threonine dehydrogenase that oxidizes threonine to 2-amino-3-oxobutyrate, which forms glycine and acetyl CoA, whereas threonine dehydratase that catabolizes threonine into 2-oxobutyrate and ammonia, is significantly less active. Increasing the threonine plasma concentrations leads to accumulation of threonine and glycine in the brain. Such accumulation affects the neurotransmitter balance which may have consequences for the brain development during early postnatal life. Thus, excessive threonine intake during infant feeding should be avoided.
Trimethylamine		Trimethylamine, is a colorless, hygroscopic, and flammable simple amine with a typical fishy odor in low concentrations and an ammonia like odor in higher concentrations. Trimethylamine is a product of decomposition of plants and animals. It is the substance mainly responsible for the fishy odor often associated with fouling fish, bacterial vagina infections, and bad breath. It is also associated with taking large doses of choline. Trimethylaminuria is a genetic disorder in which the body is unable to metabolize trimethylamine from food sources. Patients develop a characteristic fish odour of their sweat, urine, and breath after the consumption of choline-rich foods. Trimethylaminuria is an autosomal recessive disorder involving a trimethylamine oxidase deficiency.

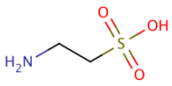
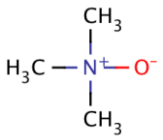
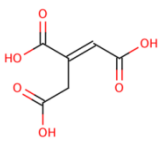
Metabolite	Formula	Main biological Function
Taurine		Taurine is a sulfur amino acid. It is a lesser-known amino acid because it is not incorporated into the structural building blocks of protein. Yet taurine is an essential amino acid in pre-term and newborn infants of humans and many other species. Adults can synthesize taurine, yet are probably dependent in part on dietary taurine. Taurine is abundant in the brain, heart, breast, gallbladder and kidney and has important roles in health and disease in these organs. Taurine has many diverse biological functions serving as a neurotransmitter in the brain, a stabilizer of cell membranes and a facilitator in the transport of ions such as sodium, potassium, calcium and magnesium. Taurine is highly concentrated in animal and fish protein, which are good sources of dietary taurine. It can be synthesized by the body from cysteine when vitamin B6 is present. Deficiency of taurine occurs in premature infants and neonates fed formula milk, and in various disease states. Inborn errors of taurine metabolism have been described. Taurine, after GABA, is the second most important inhibitory neurotransmitter in the brain. Its inhibitory effect is one source of taurine's anticonvulsant and antianxiety properties. Taurine in the brain is usually associated with zinc or manganese. The amino acids alanine and glutamic acid, as well as pantothenic acid, inhibit taurine metabolism while vitamins A and B6, zinc and manganese help build taurine. Cysteine and B6 are the nutrients most directly involved in taurine synthesis. Taurine levels have been found to decrease significantly in many depressed patients. Low levels of taurine are found in retinitis pigmentosa. Taurine deficiency in experimental animals produces degeneration of light-sensitive cells.Taurine has many important metabolic roles. Supplements can stimulate prolactin and insulin release. The parathyroid gland makes a peptide hormone called glutataurine (glutamic acid-taurine), which further demonstrates taurine's role in endocrinology. Taurine increases bilirubin and cholesterol excretion in bile, critical to normal gallbladder function. It seems to inhibit the effect of morphine and potentiates the effects of opiate antagonists. Low plasma taurine levels have been found in a variety of conditions, i.e., depression, hypertension, hypothyroidism, gout, institutionalized patients, infertility, obesity, kidney failure and others.
Trimethylamine N-oxide		TMAO is an oxidation product of trimethylamine and a common metabolite in animals and humans. In particular, trimethylamine-N-oxide is biosynthesized from trimethylamine, which is derived from choline. TMAO decomposes to trimethylamine (TMA), which is the main odorant that is characteristic of degrading seafood. TMAO is an osmolyte that the body will use to counteract the effects of increased concentrations of urea (due to kidney failure) and can be used as a biomarker for kidney problems. Fish odor syndrome or trimethylaminuria is a defect in the production of the enzyme flavin containing monooxygenase 3 causing incomplete breakdown of trimethylamine from choline-containing food into trimethylamine oxide. Trimethylamine then builds up and is released in the person's sweat, urine, and breath, giving off a strong fishy odor.
***trans*-Aconitate**		trans-Aconitic acid is normally present in normal human urine, and it has been suggested that is present in larger amounts with Reye's syndrome and organic aciduria. trans-Aconitic acid is a substrate of enzyme trans-aconitate 2-methyltransferase.

Table A5-2: Detailed Demographics of the UK population studies in Chapter 5

ID	ethnicity	age	gender	medications	diabetes
NX002	b	47	M	N	N
NX004	b	29	M	N	N
NX005	b	36	F	N	N
NX009	b	71	F	Y	N
NX011	b	34	M	N	N
NX013	b	73	F	Y	Y
NX017	b	55	M	Y	N
NX018	b	28	F	N	Y
NX019	b	51	F	Y	Y
NX022	b	28	M	N	N
NX033A	b	38	M	N	N
NX034	b	33	F	N	N
NX041	b	30	F	N	N
NX066	b	37	M		N
NX067	b	52	M	Y	Y
NX081	b	45	M		N
O14	b	44	M		N
O18	b	29	M	Y	N
O20	b	45	M	N	N
O22	b	38	M	Y	Y
O23	b	59	M	Y	N
O24	b	67	M		N
O25	b	60	M	Y	Y
O30	b	42	M	Y	N
O31	b	51	M	Y	N
O33	b	54	F	Y	Y
O38	b	49	M	Y	Y
O45	b	31	F	N	N
O46	b	72	M	Y	Y
O47	b	72	F	Y	N
O49	b		M	Y	Y
OO4	b		F	Y	Y
OO7	b	67	M	Y	N
uk 5	c	42	F	Y	N
uk 7	c	36	M	N	N
uk 8	c	22	F	Y	N
uk 9	c	24	F	N	N
uk 12	c	39	F	N	N
uk 22	c	36	F	Y	N
uk 34	c	21	F	N	N
uk 44	c	26	M	Y	N
uk 55	c	49	F	1	N
uk60	c	28	M	0	N

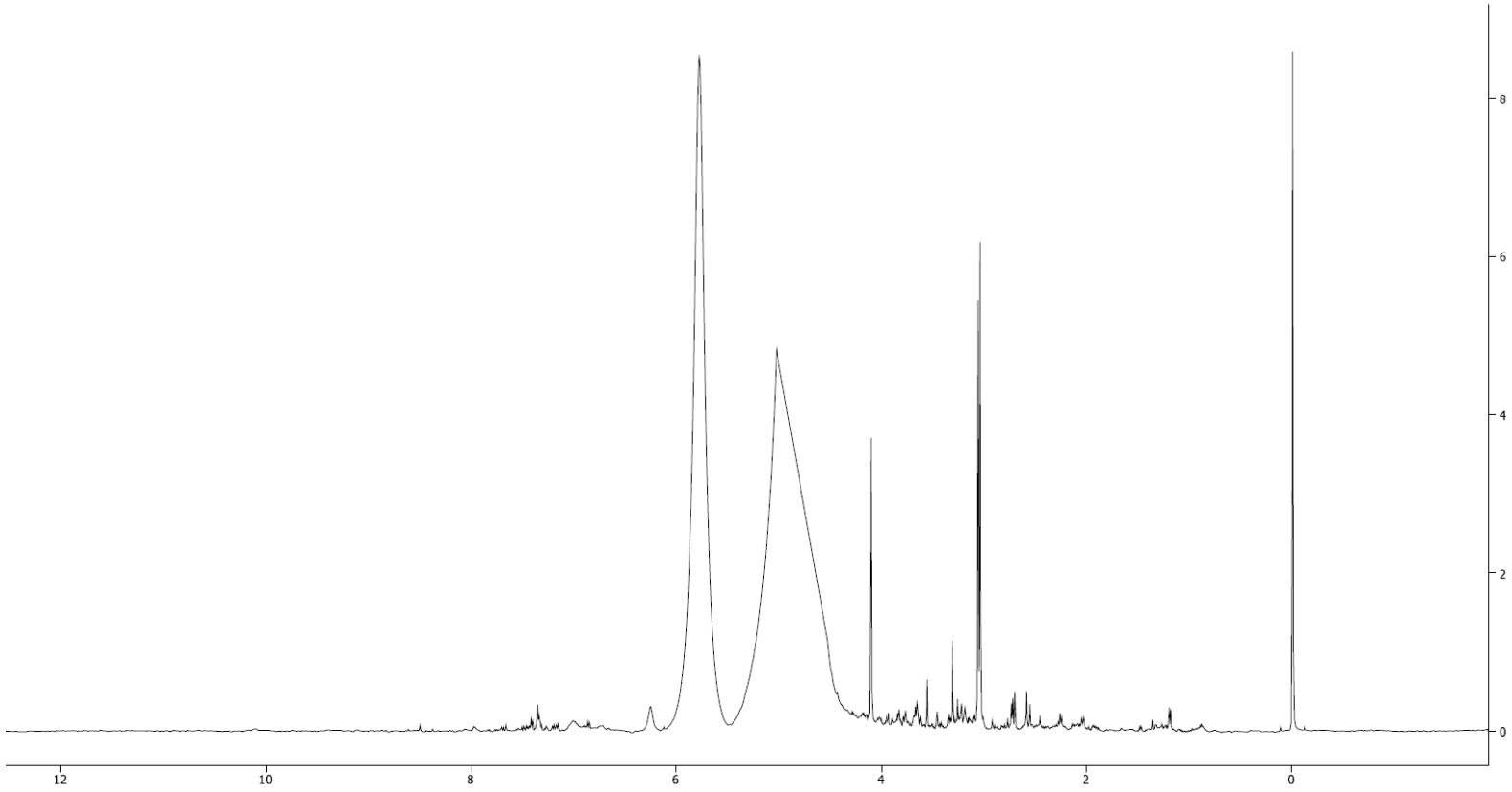


Figure A5-1: ^{1}H-NMR Spectra of urine of a UK-B volunteer (OO4)

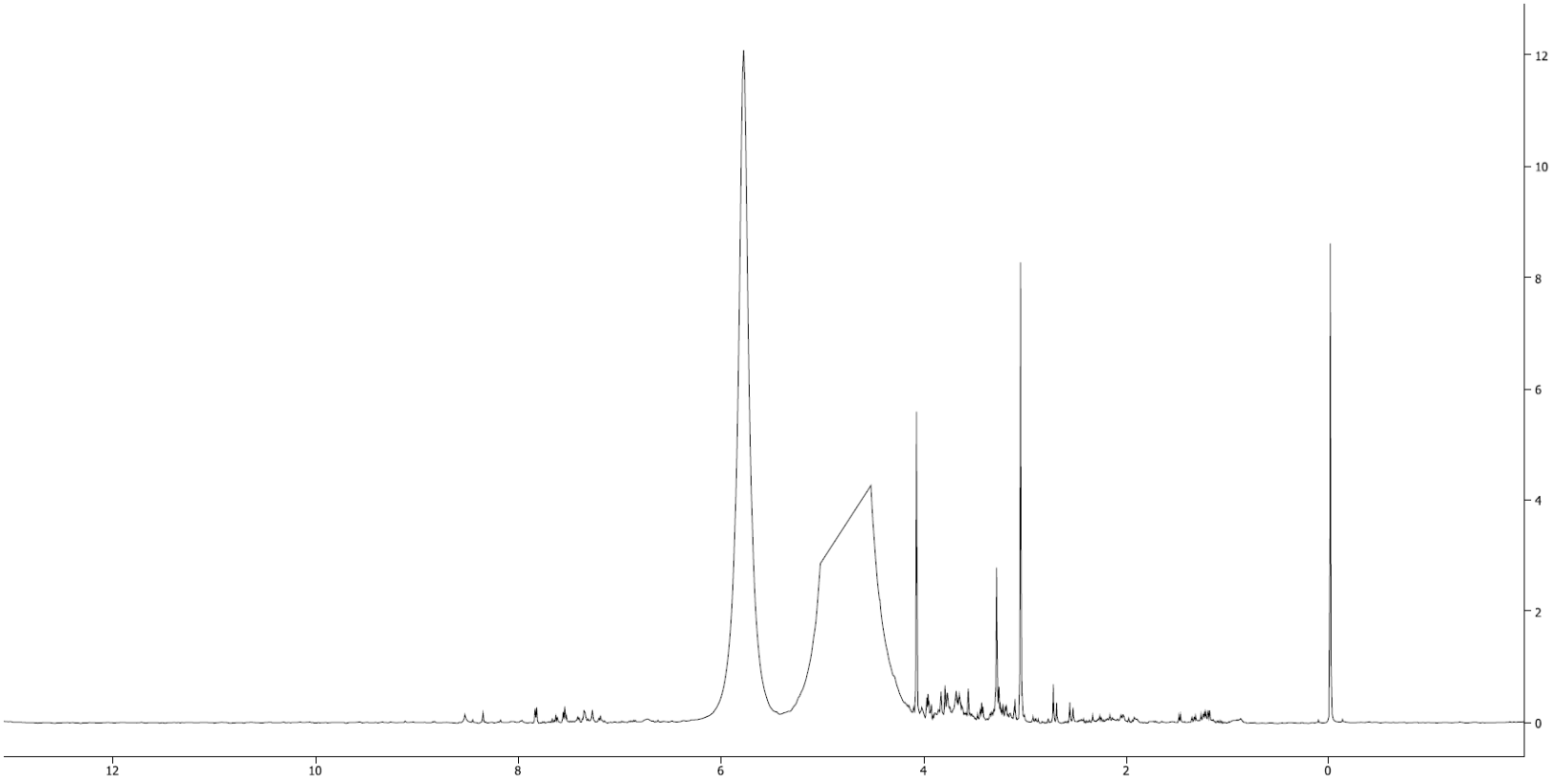


Figure A5-2: ^{1}H-NMR Spectra of urine (NIES reference material)

Appendix to Chapter 6

Table A6-1: Weekly dietary Intake for Catania [μg/Kg b. w.] (Fallico and Ferrante, 1999, Fallico and Ferrante, 2000)

	Summer	**Pb**	**Hg**	**Cd**	**Cr**	**Mn**	**Co**	**Ni**
Urban house		16.7	1.9	1.8	8.3	181.8	18.2	81.7
Geriatric House		16.9	5.6	4.8	11	237.8	23.8	84.5
University Cafeteria		23.6	2.8	3.9	9.6	255.5	25.6	90.1
Farm House		23	2.7	0.5	12.4	283.7	28.3	95.0
	Autumn							
Urban house		16.4	2	1.8	8	179.3	17.9	80.1
Geriatric House		16.7	5	3.1	10.8	235.2	21.7	80.9
University Cafeteria		23.4	2.7	3.8	9.4	251.6	23.6	88.9
Farm House		23.2	2.6	0.6	12	280.3	26.6	92.3
	Winter							
Urban house		16.1	2	2	8.2	180.2	18.1	81.0
Geriatric House		16.9	5.4	5.4	10.7	236.3	22	82.1
University Cafeteria		23.6	2.5	2.5	9.2	254.2	24.7	89.0
Farm House		23.8	2.2	2.2	12.1	281.2	27.1	93.7
	Spring							
Urban house		16	2.2	2.2	8.3	181	18.1	81.5
Geriatric House		16.7	5.2	5.2	11.1	237.1	22.9	84.3
University Cafeteria		23.5	3	3	9.5	254.9	25.2	89.7
Farm House		23.4	3.2	3.2	12.3	283.1	28.1	94.5
		Pb	**Hg**	**Cd**	**Cr**	**Mn**	**Co**	**Ni**
	average	20.0	3.2	2.9	10.2	238.3	23.2	86.8
	st dev	3.6	1.3	1.5	1.6	38.3	3.6	5.4

Table A6-2: Urinary trace element concentrations in patients affected by multiple sclerosis (A) and controls (B) [µg/L]

Sample ID	Mn	Co	Ni	Cu	Zn	Cd	Tl	Pb	Cr	As	Se
A01	1.43	0.62	3.10	15.8	200	0.554	0.18	0.954	0.35	44.9	30.1
A02	2.72	0.67	7.04	25.4	583	0.935	0.446	9.69	1.09	39.9	46.3
A05	2.25	0.65	2.56	10.4	<LOD	0.289	0.144	0.481	0.56	24.1	20.7
A06	2.28	0.18	1.98	8.4	237	0.253	0.266	0.644	0.51	43.5	18.0
A07	1.61	1.83	6.23	29.6	906	1.281	1.362	1.21	0.96	67.1	61.3
A08	2.49	0.72	3.46	22.1	280	0.329	0.83	1.40	1.12	36.3	52.9
A10	2.44	0.28	2.87	16.0	351	0.419	0.203	0.827	0.46	135.8	19.9
A100	1.46	1.43	3.82	18.3	286	0.866	0.339	0.986	0.79	21.7	49.8
A11	1.29	0.39	1.68	5.4	98	0.067	0.092	0.103	0.19	67.3	20.8
A12	1.66	0.05	0.97	8.1	48	0.085	0.081	0.460	0.38	12.8	11.4
A15	1.88	0.74	6.28	24.1	384	0.882	0.294	1.44	1.27	38.2	70.9
B101	2.54	0.21	1.63	7.0	119	0.124	0.121	0.348	0.49	13.8	13.3
B02	<LOD	0.22	2.26	10.9	342	0.337	0.685	0.862	0.56	32.7	34.6
B03	<LOD	0.11	1.48	5.7	135	0.107	0.126	0.212	0.51	21.4	10.2
B04	1.30	0.18	2.26	9.7	160	0.256	0.271	1.23	0.66	25.7	12.9
B05	4.68	0.45	10.95	30.7	1842	0.574	4.271	10.5	0.81	84.9	29.0
B09	1.17	0.17	1.64	6.2	177	0.159	0.147	2.72	0.63	16.5	15.1
B10	1.27	3.62	8.56	16.6	654	0.392	0.204	1.34	0.73	24.8	38.3
B100	2.16	0.65	7.10	22.7	625	0.871	0.297	2.72	0.91	61.8	51.1
B102	1.62	0.19	2.06	8.3	538	0.291	0.112	0.377	0.66	11.6	17.5
B11	1.40	2.13	4.66	12.4	381	0.75	0.201	0.702	0.89	43.8	42.9
B13	1.83	0.29	3.17	11.9	145	0.21	0.269	0.429	0.86	50.2	22.2

Sample ID	Mn	Co	Ni	Cu	Zn	Cd	Tl	Pb	Cr	As	Se
B15	2.53	1.73	5.28	19.0	739	0.649	0.433	0.916	1.12	57.0	46.9
B16	2.59	0.41	3.20	11.3	285	0.454	0.138	0.626	0.75	12.2	16.2
B18	1.27	0.14	1.36	5.7	41	0.167	0.046	0.040	0.51	14.0	13.9
B19	2.02	1.31	5.34	12.9	537	0.465	0.234	1.59	0.93	35.4	43.3
B21	2.12	0.22	2.23	9.4	162	0.377	0.221	2.31	0.65	26.5	24.8
B22	2.79	0.80	8.03	39.7	1855	1.473	0.389	4.71	1.24	47.3	56.8
B23	2.31	0.56	5.48	18.2	562	0.894	0.354	1.66	0.80	17.0	54.6
B24	2.04	1.15	3.43	19.5	365	1.319	0.583	0.733	1.07	47.6	45.5
B25	1.97	0.49	2.86	9.3	135	0.346	0.235	0.908	0.86	20.8	17.7
B26	1.69	0.17	1.70	7.9	264	0.295	0.147	1.40	0.57	11.5	12.3
B27	1.84	1.52	3.16	11.9	193	0.301	0.152	0.884	0.74	22.8	20.9
B29	1.92	0.53	3.23	8.8	225	0.406	0.59	0.598	0.85	27.6	37.9
B30	<LOD	0.37	4.72	19.7	611	1.262	0.153	4.39	0.79	20.2	33.6
B33	<LOD	0.08	0.59	3.4	88	0.068	0.115	0.452	0.43	15.6	10.7
B34	<LOD	0.19	1.86	4.9	155	0.069	0.167	0.480	0.67	12.7	14.6
B39	<LOD	0.44	2.90	8.3	298	0.529	0.107	0.374	0.85	30.1	13.3
B40	2.06	0.60	4.51	18.1	371	0.191	0.23	1.85	1.14	33.0	23.1
B41	1.89	0.44	3.26	13.2	506	0.523	0.744	0.855	0.90	20.9	35.0
B42	1.89	0.38	4.00	15.8	233	0.458	0.282	6.36	0.94	23.2	49.0
B48	2.22	0.44	4.89	11.7	153	0.387	0.256	1.74	0.87	43.3	25.7
B49	3.01	1.91	9.71	37.9	598	1.057	0.319	0.959	1.12	33.3	57.2
B50	2.52	0.30	5.37	17.4	351	0.427	0.495	1.89	1.98	28.2	47.2
B54	1.89	0.30	3.47	14.3	95	0.398	0.785	1.03	0.73	31.3	34.9

Sample ID	Mn	Co	Ni	Cu	Zn	Cd	Tl	Pb	Cr	As	Se
B55	2.60	0.28	2.19	12.5	363	0.242	0.292	1.01	0.86	773.2	79.7
B56	2.34	0.16	1.79	8.4	147	0.341	0.08	0.851	0.66	27.8	19.8
B58	1.96	0.25	2.05	12.7	280	0.429	0.252	0.678	0.64	46.5	63.7
B61	2.44	1.11	7.27	13.0	537	0.265	0.232	1.26	1.07	36.0	39.0

Table A6-3: Urinary trace element concentrations in patients affected by multiple sclerosis (A) and controls (B) [µg/L] corrected by SG

SAMPLE ID	Mn	Co	Ni	Cu	Zn	Cd	Tl	Pb	Cr	As	Se
A01	1.69	0.74	3.69	18.75	238	0.66	0.21	1.13	0.42	53.3	35.7
A02	1.85	0.45	4.78	17.25	396	0.63	0.30	6.58	0.74	27.1	31.4
A05	2.14	0.61	2.44	9.85	<LOD	0.27	0.14	0.46	0.53	22.9	19.7
A06	4.81	0.37	4.18	17.80	500	0.53	0.56	1.36	1.08	91.8	37.9
A07	1.02	1.16	3.94	18.75	573	0.81	0.86	0.76	0.61	42.5	38.8
A08	1.63	0.47	2.27	14.45	184	0.22	0.54	0.92	0.73	23.8	34.6
A10	3.31	0.38	3.89	21.77	476	0.57	0.28	1.12	0.62	184.3	27.0
A100	1.07	1.04	2.79	13.34	209	0.63	0.25	0.72	0.58	15.9	36.4
A11	1.89	0.57	2.45	7.92	143	0.10	0.13	0.15	0.28	98.3	30.4
A12	5.26	0.16	3.06	25.61	152	0.27	0.26	1.46	1.19	40.6	35.9
A15	1.23	0.48	4.11	15.80	251	0.58	0.19	0.94	0.83	25.0	46.4
B101	4.82	0.39	3.10	13.34	226	0.24	0.23	0.66	0.93	26.3	25.3
B02	0.31	0.18	1.79	8.61	271	0.27	0.54	0.68	0.44	25.9	27.4
B03	0.40	0.07	0.94	3.58	85	0.07	0.08	0.13	0.32	13.5	6.4
B04	6.17	0.85	10.74	46.23	758	1.22	1.29	5.84	3.14	122.3	61.2
B05	3.55	0.34	8.32	23.35	1400	0.44	3.25	8.02	0.61	64.5	22.0
B09	1.71	0.24	2.40	9.04	259	0.23	0.21	3.97	0.92	24.1	22.0
B10	1.27	3.62	8.56	16.55	654	0.39	0.20	1.34	0.73	24.8	38.3
B100	1.46	0.44	4.82	15.40	424	0.59	0.20	1.84	0.62	41.9	34.7
B101	0.27	0.03	0.34	1.37	89	0.05	0.02	0.06	0.11	1.9	2.9
B11	1.57	2.38	5.21	13.88	425	0.84	0.22	0.78	0.99	48.9	48.0

SAMPLE ID	Mn	Co	Ni	Cu	Zn	Cd	Tl	Pb	Cr	As	Se
B13	2.05	0.33	3.55	13.31	162	0.23	0.30	0.48	0.96	56.1	24.8
B15	2.53	1.73	5.28	18.98	739	0.65	0.43	0.92	1.12	57.0	46.9
B16	3.28	0.51	4.05	14.34	361	0.58	0.17	0.79	0.94	15.4	20.5
B18	3.01	0.34	3.22	13.52	97	0.40	0.11	0.03	1.21	33.2	33.0
B19	2.56	1.66	6.77	16.33	680	0.59	0.30	2.02	1.17	44.9	54.8
B21	1.75	0.18	1.84	7.78	134	0.31	0.18	1.91	0.54	21.9	20.5
B22	1.83	0.53	5.26	26.02	1215	0.97	0.25	3.08	0.81	31.0	37.2
B23	2.31	0.56	5.48	18.22	562	0.89	0.35	1.66	0.80	17.0	54.6
B24	2.15	1.22	3.62	20.54	385	1.39	0.62	0.77	1.13	50.2	48.0
B25	3.74	0.93	5.42	17.69	257	0.66	0.45	1.73	1.64	39.4	33.6
B26	1.34	0.13	1.35	6.22	209	0.23	0.12	1.11	0.45	9.1	9.7
B27	1.84	1.52	3.16	11.85	193	0.30	0.15	0.88	0.74	22.8	20.9
B29	2.03	0.56	3.41	9.30	238	0.43	0.62	0.63	0.90	29.1	40.0
B30	1.10	0.59	7.47	31.21	968	2.00	0.24	6.94	1.24	31.9	53.2
B33	1.12	0.25	1.87	10.77	277	0.22	0.36	1.43	1.37	49.4	33.9
B34	1.12	0.33	3.22	8.44	268	0.12	0.29	0.83	1.16	22.0	25.1
B39	0.77	0.35	2.30	6.57	236	0.42	0.08	0.30	0.67	23.8	10.5
B40	1.70	0.49	3.72	14.97	307	0.16	0.19	1.53	0.94	27.3	19.0
B41	2.56	0.60	4.42	17.85	687	0.71	1.01	1.16	1.22	28.4	47.5
B42	2.11	0.42	4.47	17.67	260	0.51	0.32	7.11	1.05	25.9	54.8
B48	1.76	0.35	3.87	9.27	121	0.31	0.20	1.38	0.69	34.3	20.3
B49	3.01	1.91	9.71	37.92	598	1.06	0.32	0.96	1.12	33.3	57.2
B50	1.59	0.19	3.40	11.04	222	0.27	0.31	1.19	1.26	17.9	29.9

SAMPLE ID	Mn	Co	Ni	Cu	Zn	Cd	Tl	Pb	Cr	As	Se
B54	3.60	0.58	6.59	27.13	180	0.76	1.49	1.96	1.38	59.4	66.4
B55	2.35	0.25	1.98	11.35	328	0.22	0.26	0.92	0.77	699.6	72.1
B56	3.70	0.26	2.83	13.32	233	0.54	0.13	1.35	1.05	44.0	31.4
B58	2.66	0.33	2.79	17.21	381	0.58	0.34	0.92	0.87	63.1	86.4
B61	1.60	0.73	4.76	8.48	352	0.17	0.15	0.83	0.70	23.6	25.6

Table A6-4: Trace element composition of toenails for multiple sclerosis (A) cases and controls (B) [µg/g]

	V	Mn	Fe	Co	Ni	Cu	Zn	As	Se	Mo	U	Pb
A01	0.039	0.37	22.0	0.016	0.13	2.97	70	0.223	0.92	0.031	0.004	0.37
A02	0.033	0.22	13.9	0.006	0.05	3.26	95	0.100	1.12	0.030	0.006	0.27
A06	0.004	0.06	5.8	0.001	0.01	2.06	97	0.053	0.61	0.014	0.002	0.08
A07	0.04	0.16	11.2	0.01	0.05	3.04	72	0.052	0.77	0.021	0.022	0.59
A08	0.045	0.40	15.1	0.004	0.11	3.57	79	0.039	0.81	0.020	0.008	0.08
A10	0.018	6.24	0.9	0.007	0.06	0.97	59	0.422	0.20	0.003	0.010	0.08
A100	0.014	0.59	24.3	0.001	0.36	3.11	90	0.063	0.58	0.021	0.006	9.93
A101	0.009	1.10	21.7	0.096	0.26	1.49	114	1.320	0.25	0.003	0.009	11.09
A11	0.038	0.39	17.7	0.015	0.14	2.75	85	0.049	0.64	0.015	0.026	0.24
A15	0.025	0.23	6.0	0.016	0.09	3.11	95	0.040	0.78	0.026	0.009	0.08
B02	0.133	0.46	19.8	0.004	0.20	2.64	119	0.081	0.29	0.003	0.058	3.59
B03	0.057	1.05	56.4	0.013	0.15	3.09	123	0.075	0.81	0.017	0.021	0.48
B05	0.092	1.29	64.4	0.015	0.15	3.17	95	0.084	0.30	0.025	0.008	0.94
B09	0.028	0.16	11.4	0.032	0.28	2.55	67	0.103	0.58	0.009	0.013	0.83
B10	0.021	0.27	8.4	0.025	0.24	2.59	78	0.046	0.52	0.01	0.012	1.56
B100	0.019	0.30	14.4	0.004	0.08	2.78	75	0.070	0.90	0.011	0.004	0.22
B102	0.004	0.35	35.2	0.02	0.21	4.06	94	0.171	0.38	0.014	0.005	3.10
B11	0.041	0.68	18.9	0.014	0.86	3.57	166	0.112	0.93	0.008	0.015	1.48
B12	0.111	0.71	21.1	0.013	0.52	4.76	106	0.216	0.59	0.011	0.007	0.38
B13	0.026	0.23	8.7	0.043	0.01	2.08	76	0.066	0.26	0.003	0.021	3.75
B15	0.029	0.28	12.8	0.001	0.01	1.32	93	0.036	0.52	0.003	0.023	2.57
B16	0.044	1.07	40.6	0.013	0.38	3.63	117	0.075	0.71	0.025	0.013	1.07
B19	0.018	0.14	7.5	0.003	0.14	2.89	91	0.046	0.80	0.008	0.019	0.08
B22	0.033	0.33	14.6	0.016	0.05	3.12	91	0.192	0.64	0.011	0.018	0.66
B23	0.004	0.49	12.4	0.005	0.39	1.98	79	0.087	0.65	0.003	0.005	2.08
B24	0.018	0.19	13.5	0.002	0.12	2.55	86	4.071	0.61	0.003	0.016	0.43
B25	0.028	0.24	12.0	0.001	0.10	2.84	114	0.047	0.73	0.003	0.014	8.99
B26	0.013	0.22	8.7	0.003	2.24	2.30	91	0.042	0.59	0.003	0.007	0.73
B27	0.025	0.67	14.9	0.019	0.14	2.63	86	0.107	0.60	0.008	0.010	0.56
B28	0.02	0.30	28.9	0.012	0.15	2.82	111	0.067	0.87	0.007	0.010	0.08
B29	0.016	0.12	8.0	0.008	0.86	2.04	77	0.068	0.75	0.007	0.024	0.38
B30	0.016	0.32	19.4	0.003	0.34	8.88	85	0.051	0.39	0.003	0.006	4.59
B31	0.233	0.23	14.7	0.003	0.11	2.15	79	0.105	0.93	0.05	0.047	0.88
B32	0.034	0.55	15.9	0.006	0.04	4.02	85	0.062	0.80	0.008	0.016	0.39
B33	0.037	0.55	21.9	0.009	0.26	2.96	95	0.183	0.96	0.008	0.013	1.18
B34	0.034	2.90	21.7	0.012	0.40	4.22	95	0.104	0.56	0.013	0.010	5.79
B40	0.047	0.97	41.0	0.031	0.08	3.93	101	0.093	0.87	0.015	0.009	1.87
B41	0.022	0.34	11.8	0.004	0.07	3.38	95	0.065	0.68	0.007	0.010	0.29

	V	Mn	Fe	Co	Ni	Cu	Zn	As	Se	Mo	U	Pb
B42	0.034	0.45	23.6	0.006	0.07	4.13	99	0.069	0.77	0.011	0.011	6.44
B48	0.012	0.17	6.2	0.001	0.01	2.75	90	0.122	0.57	0.003	0.013	0.08
B49	0.038	0.20	8.0	0.003	1.11	2.48	90	0.029	0.83	0.008	0.018	0.08
B50	0.04	0.09	5.2	0.001	0.15	3.73	109	0.059	0.94	0.008	0.015	0.08
B54	0.069	0.82	24.7	0.009	0.45	2.83	75	0.058	0.93	0.01	0.014	0.93
B55	0.12	1.55	38.0	0.016	0.18	6.00	152	0.132	2.07	0.019	0.034	1.82
B56	0.041	0.81	23.2	0.016	0.18	3.26	117	0.063	0.72	0.015	0.014	0.59
B58	0.034	0.31	15.7	0.006	0.07	2.44	76	0.084	0.83	0.007	0.022	0.31
B61	0.045	0.38	11.4	0.011	0.62	3.23	119	0.092	0.79	0.011	0.017	0.35

Table A6-5: Trace elements in water of 4 sampling sites in the Linguaglossa area analysed by ICP-MS at the University of Manchester

Trace elements in the groundwater	average	SD
Element	**ppb**	**ppb**
51V	29.32	6.19
52Cr	2.29	0.51
55Mn	0.09	0.81
59Co	0.14	0.02
60Ni	0.42	1.34
65Cu	6.20	10.14
66Zn	25.37	26.44
75As	2.00	0.28
82Se	0.85	0.11
111Cd	0.15	0.11
137Ba	5.53	2.23
208Pb	0.03	0.33

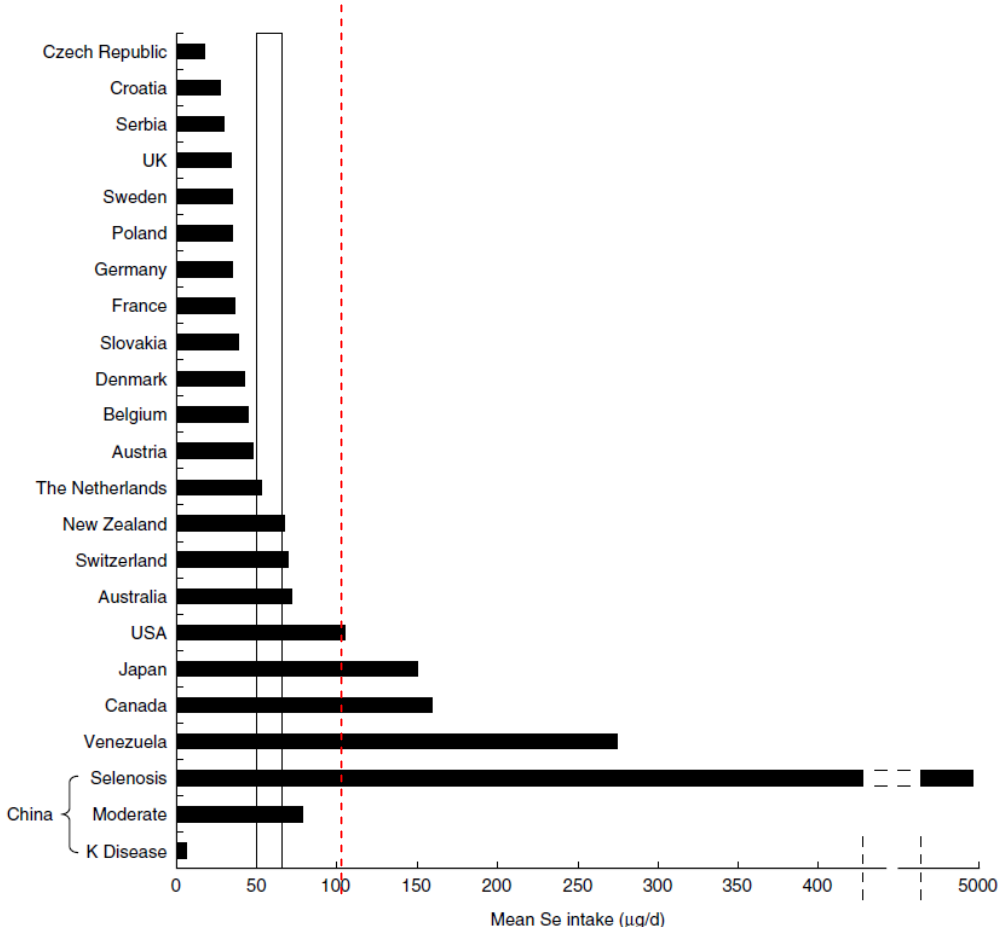


Figure 6A-1: Mean selenium intake levels µg/day. different countries as reported by (Rayman, 2005). The red dotted line represents the estimated selenium intake for Italy as from (Lombardi-Boccia *et al.*, 2003) of 103.6 µg/day.

Appendix to Chapter 7

Table A6.1 : trace element composition of Cerebrospinal fluid

ID	LABEL	Al	Mn	Co	Ni	Cu	Zn	Cd	Pb	As	Se	Mo
CNT1	CNT	54.9	1.119	0.074	1.583	21.16	13.36	0.068	0.485	<LOD	8.2	0.297
CNT2	CNT	2.0	1.049	0.056	1.814	13.81	6.363	<LOD	0.08	<LOD	1.99	0.425
CNT3	CNT	2.5	1.601	0.083	1.754	16.04	8.8	<LOD	0.531	<LOD	2.923	0.335
CNT4	CNT	10.9	1.309	0.095	2.339	10.5	27.98	<LOD	0.103	2.20	2.036	0.376
CNT5	CNT	8.2	3.363	0.181	6.461	20.94	19.97	0.02	0.377	<LOD	2.415	1.093
CNT6	CNT	1.9	2.729	0.17	4.153	18.05	7.979	<LOD	0.108	<LOD	3.395	0.275
CNT7	CNT	1.4	1.54	0.096	1.873	12.6	<LOD	<LOD	0.046	<LOD	2.545	0.19
CNT8	CNT	2.0	1.774	0.103	2.08	16.92	8.666	<LOD	0.088	<LOD	2.193	0.232
CNT9	CNT	2.2	1.472	0.072	1.791	8.785	<LOD	<LOD	0.184	<LOD	3.53	0.364
CNT10	CNT	1.0	1.327	0.079	2.867	10.83	<LOD	<LOD	0.241	<LOD	17.54	0.107
CNT11	CNT	15.6	1.377	0.071	1.823	17.25	<LOD	<LOD	0.068	<LOD	1.526	0.366
CNT13	CNT	1.7	1.137	0.089	1.818	11.87	<LOD	<LOD	0.092	<LOD	69.39	0.266
CNT14	CNT	1.5	1.527	0.077	1.98	17.44	<LOD	<LOD	0.061	<LOD	6.031	0.35
CSF 01	MS	3.9	1.409	0.087	1.857	32.14	17.3	<LOD	1.327	6.70	2.341	0.737
CSF 02	MS	24.4	0.833	0.052	0.714	13	14.74	0.088	0.253	9.60	1.39	0.54
CSF 03	MS	2.2	2.512	0.136	3.524	14.52	20.71	<LOD	0.149	7.10	1.833	0.791
CSF 04	MS	4.7	2.942	0.279	7.143	37.24	52.6	<LOD	0.03	8.70	1.715	0.699
CSF 05	MS	2.3	2.14	0.107	3.768	28.62	20.84	<LOD	0.611	7.10	1.312	0.653
CSF 06	MS	6.1	1.414	0.104	1.644	12.36	37.86	1.534	0.687	7.40	1.298	0.789
CSF 07	MS	3.5	6.065	0.272	13.41	11.86	8.564	0.027	0.255	7.10	1.69	0.693
CSF 08	MS	3.6	3.248	0.171	4.295	34.04	29.93	0.062	0.832	7.30	1.2	0.652
CSF 09	MS	3.0	2.42	0.172	6.931	11.84	14.87	<LOD	0.164	7.00	1.149	0.824
CSF 10	MS	3.9	1.326	0.023	0.967	23.12	21.54	0.026	0.227	7.30	2.306	0.838
CSF 11	MS	43.4	2.493	0.049	1.13	18.13	27.16	0.094	1.347	4.90	1.444	0.735
CSF 12	MS	81.2	1.945	0.065	2.537	20.7	29.48	0.101	1.101	7.20	1.765	0.89
CSF 13	MS	3.2	1.416	0.044	0.882	15.15	26.19	0.066	0.798	9.40	1.38	0.318
CSF 14	MS	15.8	1.01	0.056	2.066	27.97	23.32	0.023	0.354	5.80	2.228	0.35
CSF 15	MS	3.9	5.597	0.284	16.73	17.26	42.42	0.026	0.355	7.20	3.744	0.511
CSF 16	MS	12.3	0.753	0.035	1.949	21.78	20.71	<LOD	0.744	2.70	1.143	0.316
CSF 18	MS	59.4	0.982	0.042	6.787	12.46	25.01	<LOD	0.059	7.80	1.447	0.299
CSF 19	MS	2.9	1.007	0.062	1.554	7.724	14.74	<LOD	0.079	4.80	1.269	0.359
CSF 20	MS	22.2	2.618	0.144	2.349	39.4	34.74	0.048	0.346	7.20	2.671	1.148
CSF 21	MS	5.2	1.156	0.078	1.573	16.4	18.58	<LOD	0.147	5.40	3.046	0.397
CSF 22	MS	4.1	2.849	0.064	4.403	29.33	20.54	<LOD	0.805	8.50	1.998	0.313
CSF 23	MS	5.0	3.225	0.223	6.755	20.53	30.59	0.023	0.46	6.80	1.9	0.594
CSF 24	MS	2.6	2.051	0.131	8.71	7.733	20.48	0.022	0.766	7.50	0.862	0.276
CSF 25	MS	67.5	1.204	0.062	1.841	9.959	25.14	0.024	0.539	4.30	3.454	0.321
CSF 26	MS	10.8	3.297	0.223	6.743	48.26	56.13	0.09	0.73	<LOD	103.2	2.288
CSF 27	MS	10.4	5.504	0.161	4.674	16.6	35.29	0.187	0.559	5.40	2.361	0.303
CSF 28	MS	3.8	1.395	0.098	2.367	15.28	26.18	<LOD	0.108	3.80	25.98	0.197
MS01	MS	4.5	1.202	0.097	2.454	19.39	22.75	0.044	0.082	8.90	2.775	0.305
MS02	MS	4.4	0.852	0.076	5.757	11.84	15.82	0.02	0.736	2.60	41.43	0.253
MS03	MS	32.8	1.126	0.066	1.93	8.642	41.5	0.083	0.105	5.80	1.714	0.28
MS06	MS	29.2	1.687	0.088	3.153	22.29	50.37	0.122	0.579	6.30	4.621	0.426
MS08	MS	19.3	0.756	0.05	1.509	9.974	45.15	0.046	0.19	3.80	1.223	0.333
MS09	MS	3.1	0.942	0.058	1.952	10.51	16.73	0.022	0.035	6.20	2.326	0.435
SM04	MS	7.0	2.321	0.239	8.248	17.48	35.66	0.03	0.129	7.90	4.106	0.404
SM05	MS	8.7	0.727	0.066	1.832	10.77	39.45	0.049	0.241	6.10	1.978	0.404
SM07	MS	10.3	1.046	0.06	1.651	9.389	14.28	0.026	0.084	11.40	2.698	0.27

REFERENCES

ABBYADA, P., TROMPA, J., LAMB, J. & SALIN, E. 2001. Optimization of the technique of standard additions for inductively coupled plasma mass spectrometry. *Atomic Spectroscopy,* 16.

ABEDIN, M. J., COTTER-HOWELLS, J. & MEHARG, A. A. 2002a. Arsenic uptake and accumulation in rice (Oryza sativa L.) irrigated with contaminated water. *Plant and Soil,* 240**,** 311-319.

ABEDIN, M. J., FELDMANN, J. & MEHARG, A. A. 2002b. Uptake kinetics of arsenic species in rice plants. *Plant Physiology,* 128**,** 1120-1128.

ACAR, G., IDIMAN, F., KIRKALI, G., ÖZAKBAŞ, S., OKTAY, G., ÇAKMAKÇI, H. & IDIMAN, E. 2005. Intrathecal sICAM-1 production in multiple sclerosis Correlation with triple dose Gd-DTPA MRI enhancement and IgG index. *Journal of Neurology,* 252**,** 146-150.

ACON, B. W., MCLEAN, J. A. & MONTASER, A. 2001. A direct injection high efficiency nebulizer interface for microbore high-performance liquid chromatography-inductively coupled plasma mass spectrometry. *Journal of Analytical Atomic Spectrometry,* 16**,** 852-857.

AGILENT 2005. ICP-MS Inductively Coupled Plasma Mass Spectrometry. USA.

AGUSA, T., KUNITO, T., FUJIHARA, J., KUBOTA, R., MINH, T. B., KIM TRANG, P. T., IWATA, H., SUBRAMANIAN, A., VIET, P. H. & TANABE, S. 2006. Contamination by arsenic and other trace elements in tube-well water and its risk assessment to humans in Hanoi, Vietnam. *Environmental Pollution,* 139**,** 95-106.

AGUSA, T., KUNITO, T., MINH, T. B., KIM TRANG, P. T., IWATA, H., VIET, P. H. & TANABE, S. 2009. Relationship of urinary arsenic metabolites to intake estimates in residents of the Red River Delta, Vietnam. *Environmental Pollution,* 157**,** 396-403.

AHSAN, H., CHEN, Y., PARVEZ, F., ARGOS, M., HUSSAIN, A. I., MOMOTAJ, H., LEVY, D., VAN GEEN, A., HOWE, G. & GRAZIANO, J. 2006. Health Effects of Arsenic Longitudinal Study (HEALS): Description of a multidisciplinary epidemiologic investigation. *Journal of Exposure Science and Environmental Epidemiology,* 16**,** 191.

AIUPPA, A., AVINO, R., BRUSCA, L., CALIRO, S., CHIODINI, G., D'ALESSANDRO, W., FAVARA, R., FEDERICO, C., GINEVRA, W., INGUAGGIATO, S., LONGO, M., PECORAINO, G. & VALENZA, M. 2006. Mineral control of arsenic content in thermal waters from volcano-hosted hydrothermal systems: Insights from island of Ischia and Phlegrean Fields (Campanian Volcanic Province, Italy). *Chemical Geology,* 229**,** 313-330.

AIUPPA, A., BELLOMO, S., BRUSCA, L., D'ALESSANDRO, W. & FEDERICO, C. 2003. Natural and anthropogenic factors affecting groundwater quality of an active volcano (Mt. Etna, Italy). *Applied Geochemistry,* 18**,** 863.

AL-RMALLI, S., JENKINS, R. & HARIS, P. 2011a. Betel quid chewing as a source of manganese exposure: total daily intake of manganese in a Bangladeshi population. *BMC Public Health,* 11**,** 85.

AL-RMALLI, S. W., JENKINS, R. O. & HARIS, P. I. 2011b. Betel quid chewing elevates human exposure to arsenic, cadmium and lead. *Journal of Hazardous Materials,* 190**,** 69-74.

AL-RMALLI, S. W., JENKINS, R. O., WATTS, M. J. & HARIS, P. I. 2010. Risk of human exposure to arsenic and other toxic elements from geophagy: Trace element analysis of baked clay using inductively coupled plasma mass spectrometry. *Environmental Health: A Global Access Science Source,* 9.

AL RMALLI, S. W., HARIS, P. I., HARRINGTON, C. F. & AYUB, M. 2005. A survey of arsenic in foodstuffs on sale in the United Kingdom and imported from Bangladesh. *Science of the Total Environment,* 337**,** 23-30.

ALIMONTI, A., BOCCA B, MATTEI D, LAMAZZA A, FIORI E, DE MASI E, PINO A & G., F. 2009. Composition of essential and non-essential elements in tissues and body fluids of healthy subjects and patients with colorectal polyps. . *Int J Environ Health,* 3**,** 224-37.

ALIMONTI, A., BOCCA, B., MATTEI, D. & PINO, A. 2010. Biomonitoraggio della popolazione italiana per l'esposizione ai metalli: valori di riferimento 1990-2009. . *Rapporti ISTISAN 10/22*.

ALIMONTI, A., BOCCA, B., PINO, A., RUGGIERI, F., FORTE, G. & SANCESARIO, G. 2007a. Elemental profile of cerebrospinal fluid in patients with Parkinson's disease. *Journal of Trace Elements in Medicine and Biology,* 21**,** 234-241.

ALIMONTI, A., RISTORI, G., GIUBILEI, F., STAZI, M. A., PINO, A., VISCONTI, A., BRESCIANINI, S., MONTI, M. S., FORTE, G., STANZIONE, P., BOCCA, B., BOMBOI, G., D'IPPOLITO, C., ANNIBALI, V., SALVETTI, M. & SANCESARIO, G. 2007b. Serum chemical elements and oxidative status in Alzheimer's disease, Parkinson disease and multiple sclerosis. *NeuroToxicology,* 28**,** 450-456.

ALVES-LEON, S. V., BATISTA, E., PAPAIS-ALVARENGA, R. & QUÍRICO-SANTOS, T. 2001. Determination of soluble ICAM-1 and TNFαR in the cerebrospinal fluid and serum levels in a population of Brazilian patients with relapsing-remitting multiple sclerosis. *Arquivos de Neuro-Psiquiatria,* 59**,** 18-22.

ANDRONICO, D., SPINETTI, C., CRISTALDI, A. & BUONGIORNO, M. F. 2009. Observations of Mt. Etna volcanic ash plumes in 2006: An integrated approach from ground-based and polar satellite NOAA-AVHRR monitoring system. *Journal of Volcanology and Geothermal Research,* 180**,** 135-147.

ANGERER, J., BIRD, M. G., BURKE, T. A., DOERRER, N. G., NEEDHAM, L., ROBISON, S. H., SHELDON, L. & ZENICK, H. 2006. Strategic Biomonitoring Initiatives: Moving the Science Forward. *Toxicological Sciences,* 93**,** 3-10.

ANGERER, J., EWERS, U. & WILHELM, M. 2007. Human biomonitoring: State of the art. *International Journal of Hygiene and Environmental Health,* 210**,** 201-228.

APOSHIAN, H. V. 1997. Enzymatic methylation of arsenic species and other new approaches to arsenic toxicity. *Annual Review of Pharmacology and Toxicology.*

APPLEBAUM, K. M., KARAGAS, M. R., HUNTER, D. J., CATALANO, P. J., BYLER, S. H., MORRIS, S. & NELSON, H. H. 2007. Polymorphisms in nucleotide excision repair genes, arsenic exposure,and non-melanoma skin cancer in New Hampshire,. *Environ. Health Perspect.,* 115**,** 1231–1236.

APPLETON, J. D., RAWLINS, B. G. & THORNTON, I. 2008. National-scale estimation of potentially harmful element ambient background concentrations in topsoil using parent material classified soil:stream-sediment relationships. *Applied Geochemistry,* 23**,** 2596-2611.

ARGOS, M., KALRA, T., RATHOUZ, P. J., CHEN, Y., PIERCE, B., PARVEZ, F., ISLAM, T., AHMED, A., RAKIBUZ-ZAMAN, M., HASAN, R., SARWAR, G., SLAVKOVICH, V., VAN GEEN, A., GRAZIANO, J. & AHSAN, H. 2010. Arsenic exposure from drinking water, and all-cause and chronic-disease mortalities in Bangladesh (HEALS): a prospective cohort study. *The Lancet,* 376**,** 252-258.

ASCHERIO, A. & MUNGER, K. L. 2007. Environmental risk factors for multiple sclerosis. Part I: The role of infection. *Annals of Neurology,* 61**,** 288-299.

ASTDR 2007. TOXICOLOGICAL PROFILE FOR LEAD. *U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES* Atlanta.

ATSDR 1993. Toxicological profile for arsenic.

ATSDR 2001. Hair Analysis Panel Discussion: Exploring the State of the Science. Atlanta.

ATSDR 2005. TOXICOLOGICAL PROFILE FOR NICKEL. Atlanta, Georgia: Agency for Toxic Substances and Disease Registry.

ATSDR 2007. Toxicological profile for arsenic. . Atlanta, GA: U. S. Department of Health and Human Services, Public Health Service.

AU, W.-Y., TAM, S., FONG, B. M. & KWONG, Y.-L. 2006. Elemental arsenic entered the cerebrospinal fluid during oral arsenic trioxide treatment of meningeal relapse of acute promyelocytic leukemia. *Blood,* 107**,** 3012-3013.

AZIZUR RAHMAN, M., HASEGAWA, H., MAHFUZUR RAHMAN, M., MAZID MIAH, M. A. & TASMIN, A. 2008. Arsenic accumulation in rice (Oryza sativa L.): Human exposure through food chain. *Ecotoxicology and Environmental Safety,* 69**,** 317-324.

BAGNATO, E., AIUPPA, A., PARELLO, F., CALABRESE, S., D'ALESSANDRO, W., MATHER, T. A., MCGONIGLE, A. J. S., PYLE, D. M. & WÄNGBERG, I. 2007. Degassing of gaseous (elemental and reactive) and particulate mercury from Mount Etna volcano (Southern Italy). *Atmospheric Environment,* 41**,** 7377-7388.

BAKER, S. A., BRADSHAW, D. K. & MILLER-IHLI, N. J. 1999. Trace element determinations in food and biological samples using ICP-MS. *Atomic Spectroscopy,* 20**,** 167-173.

BANERJEE, M., SARKAR, J., DAS, J. K., MUKHERJEE, A., SARKAR, A. K., MONDAL, L. & GIRI, A. K. 2007. Polymorphism in the ERCC2 codon 751 is associated with arsenic-induced premalignant hyperkeratosis and significant chromosome aberrations. *Carcinogenesis,* 28 672-676.

BANNING, A., COLDEWEY, W. G. & GÃ¶BEL, P. 2008a. A procedure to identify natural arsenic sources, applied in an affected area in North Rhine-Westphalia, Germany. *Environmental Geology***,** 1-13.

BANNING, A., COLDEWEY, W. G. & GO?BEL, P. 2008b. A procedure to identify natural arsenic sources, applied in an affected area in North Rhine-Westphalia, Germany. *Environmental Geology***,** 1-13.

BANZA, C. L. N., NAWROT, T. S., HAUFROID, V., DECRÉE, S., DE PUTTER, T., SMOLDERS, E., KABYLA, B. I., LUBOYA, O. N., ILUNGA, A. N., MUTOMBO, A. M. & NEMERY, B. 2009. High human exposure to cobalt and other metals in Katanga, a mining area of the Democratic Republic of Congo. *Environmental Research,* 109**,** 745-752.

BARBA, C., CAVALLI-SFORZA, T., CUTTER, J., DARNTON-HILL, I., DEURENBERG, P., DEURENBERG-YAP, M., GILL, T., JAMES, P., KO, G. & NISHIDA, C. 2004. Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies. *Lancet,* 363**,** 157.

BARBANEL, C. S., WINKELMAN, J. W., FISCHER, G. A. & KING, A. J. 2002. Confirmation of the department of transportation criteria for a substituted urine specimen. *Journal of Occupational and Environmental Medicine,* 44**,** 407-416.

BARCELOUX, D. 1999. Cobalt. *J Toxicol Clin Toxicol,* 37**,** 201-6.

BARR, D. B., WILDER, L. C., CAUDILL, S. P., GONZALEZ, A. J., NEEDHAM, L. L. & PIRKLE, J. L. 2005. Urinary creatinine concentrations in the U.S. population: Implications for urinary biologic monitoring measurements. *Environmental Health Perspectives,* 113**,** 192-200.

BARRATT, J. M. B. C. & TOPHAM, P. M. B. C. M. D. 2007. Urine proteomics: the present and future of measuring urinary protein components in disease. *CMAJ,* 177**,** 361-368.

BARSOTTI, S., ANDRONICO, D., NERI, A., DEL CARLO, P., BAXTER, P. J., ASPINALL, W. P. & HINCKS, T. 2010. Quantitative assessment of volcanic ash hazards for health and infrastructure at Mt. Etna (Italy) by numerical simulation. *Journal of Volcanology and Geothermal Research,* 192**,** 85-96.

BASU, A., MAHATA, J., ROY, A. K., SARKAR, J. N., PODDAR, G., NANDY, A. K., SARKAR, P. K., DUTTA, P. K., BANERJEE, A., DAS, M., RAY, K., ROYCHAUDHURY, S., NATARAJAN, A. T., NILSSON, R. & GIRI, A. K. 2002. Enhanced frequency of micronuclei in individuals exposed to arsenic through drinking water in West Bengal, India. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* 516**,** 29.

BATISTA, B. L., RODRIGUES, J. L., TORMEN, L., CURTIUS, A. J. & BARBOSA, F. 2009. Reference Concentrations for Trace Elements in Urine for the Brazilian Population based on q-ICP-MS with a Simple Dilute-and-Shoot Procedure. *Journal of Brazialian Chemical Society,* 20**,** 1406-1413.

BAXMANN, A. C., AHMED, M. S., MARQUES, N. C., MENON, V. B., PEREIRA, A. B., KIRSZTAJN, G. M. & HEILBERG, I. P. 2008. Influence of Muscle Mass and Physical Activity on Serum and Urinary Creatinine and Serum Cystatin C. *Clin J Am Soc Nephrol,* 3**,** 348-354.

BEAUCHEMIN, D. 2002. Inductively coupled plasma mass spectrometry. *Analytical Chemistry,* 74**,** 2873-2893.

BECKER, K., KOLOSSA-GEHRING, M., SEIWERT, M., CASTELEYN, L., POLCHER, A., SEPAI, O., KNUDSEN, L., SCHOETERS, G., SMOLDERS, R., CASTANO, A., JIMENEZ GUERRERO, J. A., HORVAT, M., BLOEMEN, L., ANGERER, J., KOCH, H. & JOAS, R. 2011. Exploring Exposure in 27 Countries in a European Human Biomonitoring Study-Cophes. *Epidemiology,* 22**,** S230-S231.

BENCKO, V. & SYMON, K. 1977. Test of environmental exposure to arsenic and hearing changes in exposed children. *Environmental Health Perspectives,* Vol.19**,** 95.

BENRAMDANE, L., ACCOMINOTTI, M., FANTON, L., MALICIER, D. & VALLON, J.-J. 1999. Arsenic speciation in human organs following fatal arsenic trioxide poisoning - A case report. *Clinical Chemistry,* 45 301-306.

BERG, M., TRAN, H. C., NGUYEN, T. C., PHAM, H. V., SCHERTENLEIB, R. & GIGER, W. 2001. Arsenic contamination of groundwater and drinking water in Vietnam: A human health threat. *Environmental Science and Technology,* 35**,** 2621-2626.

BERGOMI, M., VINCETI, M., NACCI, G., PIETRINI, V., BRÄTTER, P., ALBER, D., FERRARI, A., VESCOVI, L., GUIDETTI, D., SOLA, P., MALAGU, S., ARAMINI, C. & VIVOLI, G. 2002. Environmental Exposure to Trace Elements and Risk of Amyotrophic Lateral Sclerosis: A Population-Based Case-Control Study. *Environmental Research,* 89**,** 116.

BERRAR, D., GRANZOW, M. & DUBITZKY, W. 2007. Introduction to Genomic and Proteomic Data Analysis. *In:* DUBITZKY, W., GRANZOW, M. & BERRAR, D. (eds.) *Fundamentals of Data Mining in Genomics and Proteomics.* Springer US.

BERRY, M. J. A. & LINOFF, G. (eds.) 1997. *Data Mining Techniques: For Marketing, Sales, and Customer Support,* USA.

BHOPAL, R. 2004. Glossary of terms relating to ethnicity and race: for reflection and debate. *J Epidemiol Community Health,* 58**,** 441-445.

BHOPAL, R. 2009. Medicine and public health in a multiethnic world. *Journal of Public Health,* 31**,** 315-321.

BHOPAL, R., RAHEMTULLA, T. & SHEIKH, A. 2005. Persistent high stroke mortality in Bangladeshi populations. *BMJ,* 331**,** 1096-1097.

BHOPAL, R., UNWIN, N., WHITE, M., YALLOP, J., WALKER, L., ALBERTI, K. G. M. M., HARLAND, J., PATEL, S., AHMAD, N., TURNER, C., WATSON, B., KAUR, D., KULKARNI, A., LAKER, M. & TAVRIDOU, A. 1999. Heterogeneity of coronary heart disease risk factors in Indian, Pakistani, Bangladeshi, and European origin populations: Cross sectional study. *British Medical Journal,* 319**,** 215.

BIRRINGER, M., PILAWA, S. & FLOHÉ, L. 2002. Trends in selenium biochemistry. *Natural Product Reports,* 19**,** 693-718.

BISWAS, D., BANERJEE, M., SEN, G., DAS, J. K., BANERJEE, A., SAU, T. J., PANDIT, S., GIRI, A. K. & BISWAS, T. 2008. Mechanism of erythrocyte death in human population exposed to arsenic through drinking water. *Toxicology and Applied Pharmacology,* 230**,** 57–66.

BODWELL, J. E., KINGSLEY, L. A. & HAMILTON, J. W. 2004. Arsenic at very low concentrations alters glucocorticoid receptor (GR)-mediated gene activation but not GR-mediated gene repression: Complex dose-response effects are closely correlated with levels of activated GR and require a functional GR DNA binding domain. *Chemical Research in Toxicology,* 17**,** 1064-1076.

BOELLMANN, F., ZHANG, L., CLEWELL, H. J., SCHROTH, G. P., KENYON, E. M., ANDERSEN, M. E. & THOMAS, R. S. 2010. Genome-wide analysis of DNA methylation and gene expression changes in the mouse lung following subchronic arsenate exposure. *Toxicological Sciences,* 117**,** 404-417.

BOTHWELL, J. H. F. & GRIFFIN, J. L. 2010. An introduction to biological nuclear magnetic resonance spectroscopy. *Biological Reviews,* 86**,** 493.

BOWLER, R. M., GYSENS, S., DIAMOND, E., NAKAGAWA, S., DREZGIC, M. & ROELS, H. A. 2006. Manganese exposure: Neuropsychological and neurological symptoms and effects in welders. *NeuroToxicology,* 27**,** 315.

BOYCE, C. P., LEWIS, A. S., SAX, S. N., ELDAN, M., COHEN, S. M. & BECK, B. D. 2008. Probabilistic Analysis of Human Health Risks Associated with Background Concentrations of Inorganic Arsenic: Use of a Margin of Exposure Approach. *Human and Ecological Risk Assessment,* 14 1159-1201.

BRETON, C. V., ZHOU, W., KILE, M. L., HOUSEMAN, E. A., QUAMRUZZAMAN, Q., RAHMAN, M., MAHIUDDIN, G. & CHRISTIANI, D. C. 2007. Susceptibility to arsenic-induced skin lesions from polymorphisms in base excision repair genes, . *Carcinogenesis,* 28**,** 1520-1525.

BRIMA, E. I., HARIS, P. I., JENKINS, R. O., POLYA, D. A., GAULT, A. G. & HARRINGTON, C. F. 2006a. Understanding arsenic metabolism through a comparative study of arsenic levels in the urine, hair and fingernails of healthy volunteers from three unexposed ethnic groups in the United Kingdom. *Toxicology and Applied Pharmacology,* 216**,** 122-130.

BRIMA, E. I., HARIS, P. I., JENKINS, R. O., POLYA, D. A., GAULT, A. G. & HARRINGTON, C. F. 2006b. Understanding arsenic metabolism through a comparative study of arsenic levels in the

urine, hair and fingernails of healthy volunteers from three unexposed ethnic groups in the United Kingdom. *Toxicology and Applied Pharmacology,* 216**,** 122.

BRIMA, E. I., JENKINS, R. O. & HARIS, P. I. 2006c. Understanding arsenic metabolism through spectroscopic determination of arsenic in human urine. *Spectroscopy,* 20 125-151.

BRINDLE, K. M. & RADDA, G. K. 1985. Measurements of exchange in the reaction catalysed by creatine kinase using 14C and 15N isotope labels and the NMR technique of saturation transfer. *Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology,* 829**,** 188-201.

BROCKMAN, J. & SCHELL, L. 2011. A radiochemical method for neutron activation analysis of arsenic in biological samples and its potential use in epidemiology studies. *Journal of Radioanalytical and Nuclear Chemistry***,** 1-6.

BROOKINS, D. G. 1988. Eh-pH diagrams for geochemistry.

BRUNI, B. M., PACELLA, A., MAZZIOTTITAGLIANI, A., GIANFAGNA & PAOLETTI 2006. Nature and extent of the exposure to fibrous amphiboles in Biancavilla. *Science of the Total Environment* 370**,** 9-16.

BUCHET, J. P., LAUWERYS, R. & ROELS, H. 1981. Comparison of the urinary excretion of arsenic metabolites after a single oral dose of sodium arsenite, monomethylarsonate, or dimethylarsinate in man. *International Archives of Occupational and Environmental Health,* 48**,** 71-79.

BUNDSCHUH, J., FARIAS, B., MARTIN, R., STORNIOLO, A., BHATTACHARYA, P., CORTES, J., BONORINO, G. & ALBOUY, R. 2004. Groundwater arsenic in the Chaco-Pampean Plain, Argentina: case study from Robles county, Santiago del Estero Province. *Applied Geochemistry,* 19**,** 231.

BUTTON, M., JENKIN, G. R. T., HARRINGTON, C. F. & WATTS, M. J. 2009. Human toenails as a biomarker of exposure to elevated environmental arsenic. *Journal of Environmental Monitoring,* 11**,** 610-617.

C ´ AVARA, S., KLAPECB, T., JURIS˘IC´ GRUBES˘IC´C, R. & VALEKA, M. 2005. High exposure to arsenic from drinking water at several localities in eastern Croatia. *Science of the Total Environment* 339 277- 282.

CALABRESE, S. 2008. *Athmospheric deposition of volcanogenic major and trace elements on Mt. Etna, Sicily.* Palermo.

CALDERON, J., NAVARRO, M. E., JIMENEZ-CAPDEVILLE, M. E., SANTOS-DIAZ, M. A., GOLDEN, A., RODRIGUEZ-LEYVA, I., BORJA-ABURTO, V. & DAZ-BARRIGA, F. 2001. Exposure to arsenic and lead and neuropsychological development in Mexican children. *Environmental Research,* 85**,** 69-76.

CALDWELL, K. L., JONES, R. L., VERDON, C. P., JARRETT, J. M., CAUDILL, S. P. & OSTERLOH, J. D. 2009. Levels of urinary total and speciated arsenic in the US population: National Health and Nutrition Examination Survey 2003-2004. *Journal of Exposure Science and Environmental Epidemiology,* 19**,** 59.

CAMACHO, L. M., GUTIÉRREZ, M., ALARCÓN-HERRERA, M. T., VILLALBA, M. D. L. & DENG, S. Occurrence and treatment of arsenic in groundwater and soil in northern Mexico and southwestern USA. *Chemosphere,* 83**,** 211.

CANFIELD, W. K., HAMBIDGE, K. M. & JOHNSON, L. A. K. 1984. Zinc nutriture in type I diabetes mellitus: Relationship to growth measures and metabolic control. *Journal of Pediatric Gastroenterology and Nutrition,* 3**,** 577-584.

CARTER, J. M., SANDO, S. K., HAYES, T. S. & HAMMOND, R. H. 1998. Source Occurrence, and Extent of Arsenic Contamination in the Grass Mountain Area of the Rosebud Indian Reservation, South Dakota. . *In:* 97-4286., U. W. R. I. R. (ed.).

CARUSO, J. A. & MONTES-BAYON, M. 2003. Elemental speciation studies - New directions for trace metal analysis. *Ecotoxicology and Environmental Safety,* 56**,** 148-163.

CASCIO, C., RAAB, A., JENKINS, R. O., FELDMANN, J., MEHARG, A. A. & HARIS, P. I. 2011. The impact of a rice based diet on urinary arsenic. *Journal of Environmental Monitoring,* 13**,** 257-265.

CASTANO ESTEBAN M 2009. A: Non-invasive matrices in human biomonitoring: A review. *Environ Internat* 35**,** 438-449.

CHAKRABORTI, D., MUKHERJEE, S. C., PATI, S., SENGUPTA, M. K., RAHMAN, M. M., CHOWDHURY, U. K., LODH, D., CHANDA, C. R., CHAKRABORTI, A. K. & BASU, G. K. 2003. Arsenic groundwater

contamination in Middle Ganga Plain, Bihar, India: A future danger? *Environmental Health Perspectives,* 111**,** 1194-1201.

CHAMBERS, J. C., OBEID, O. A., REFSUM, H., UELAND, P., HACKETT, D., HOOPER, J., TURNER, R. M., THOMPSON, S. G. & KOONER, J. S. 2000. Plasma homocysteine concentrations and risk of coronary heart disease in UK Indian Asian and European men. *Lancet,* 355**,** 523.

CHANDRA, R. K. 1984. Excessive Intake of Zinc Impairs Immune Responses. *JAMA: The Journal of the American Medical Association,* 252**,** 1443-1446.

CHANDRA, S. K., CHARY, N. S., KAMALA, C. T., VENKATESWARA, R. J., BALARAM, V. & ANJANEYULU, Y. 2003. Risk assessment and pathway study of arsenic in industrially contaminated sites of Hyderabad: a case study. *Environment International,* 29**,** 601.

CHARLET, L. & POLYA, D. A. 2006. Arsenic in shallow, reducing groundwaters in Southern Asia: An environmental health disaster. *Elements,* 2**,** 91-96.

CHAUDHURI, D. S., MAHATA, J., DAS, J. K., MUKHERJEE, A., GHOSH, P., SAU, T. J., MONDAL, L., BASU, S., GIRI, A. K. & ROYCHOUDHURY, S. 2006. Association of specific p53 polymorphisms with keratosis in individuals exposed to arsenic through drinking water in West Bengal, India. *Mutat. Res.,* 601**,** 102-112.

CHAUDHURI, S. D., GHOSH, P., SARM, S., MAJUMDAR, P., SAU, T. J., BASU, S., ROYCHOUDHURY, S., RAY, K. & GIRI, A. K. 2008. Genetic variants associated with arsenic susceptibility:study of purine nucleoside phosphorylase, arsenic (+3) methyl transferase and glutathione S-transferase omega genes. *Environ. Health Perspect.,* 116**,** 501–505.

CHEN, C.-J., WANG, S.-L., CHIOU, J.-M., TSENG, C.-H., CHIOU, H.-Y., HSUEH, Y.-M., CHEN, S.-Y., WU, M.-M. & LAI, M.-S. 2007a. Arsenic and diabetes and hypertension in human populations: A review. *Toxicology and Applied Pharmacology,* 222 298-304.

CHEN, C. J., CHUANG, Y. C., LIN, T. M. & WU, H. Y. 1985. Malignant neoplasms among residents of a blackfoot disease-endemic area in Taiwan: High-arsenic artesian well water and cancers. *Cancer Research,* 45**,** 5895-5899.

CHEN, C. J., WU, M. M., LEE, S. S., WANG, J. D., CHENG, S. H. & WU, H. Y. 1988. Atherogenicity and carcinogenicity of high-arsenic artesian well water. Multiple risk factors and related malignant neoplasms of blackfoot disease. *Arteriosclerosis,* 8**,** 452-60.

CHEN, C. Y., WANG, Y. F., HUANG, W. R. & HUANG, Y. T. 2003a. Nickel induces oxidative stress and genotoxicity in human lymphocytes. *Toxicology and Applied Pharmacology,* 189**,** 153-159.

CHEN, H., LI, S. F., LIU, J., DIWAN, B. A., BARRETT, J. C. & WAALKES, M. P. 2004. Chronic inorganic arsenic exposure induces hepatic global and individual gene hypomethylation: Implications for arsenic hepatocarcinogenesis. *Carcinogenesis,* 25**,** 1779-1786.

CHEN, Y., AHSAN, H., SLAVKOVICH, V., PELTIER, G. L., GLUSKIN, R. T., PARVEZ, F., LIU, X. & GRAZIANO, J. H. 2010. No Association between Arsenic Exposure from Drinking Water and Diabetes Mellitus: A Cross-Sectional Study in Bangladesh. *Environ Health Perspect,* 118.

CHEN, Y., HALL, M., GRAZIANO, J. H., SLAVKOVICH, V., VAN GEEN, A., PARVEZ, F. & AHSAN, H. 2007b. A prospective study of blood selenium levels and the risk of arsenic-related premalignant skin lesions. *Cancer Epidemiology Biomarkers and Prevention,* 16**,** 207.

CHEN, Y. C., GUO, Y. L., SU, H. J., HSUEH, Y. M., SMITH, T. J., RYAN, L. M., LEE, M. S., CHAO, S. C., LEE, J. Y. & CHRISTIANI, D. C. 2003b. Arsenic methylation and skin cancer risk in southwestern Taiwan. *J Occup Environ Med,* 45**,** 241-8.

CHIA, Y. Y. & TON, S. H. 2006. Urinary Amino Acids Profile of Vegetarians and Non-vegetarians. *Ma1 J Nutr,* 12**,** 67-78.

CHOWDHURY, U. K., RAHMAN, M. M., SENGUPTA, M. K., LODH, D., CHANDA, C. R., ROY, S., QUAMRUZZAMAN, Q., TOKUNAGA, H., ANDO, M. & CHAKRABORTI, D. 2003. Pattern of Excretion of Arsenic Compounds [Arsenite, Arsenate, MMA(V), DMA(V)] in Urine of Children Compared to Adults from an Arsenic Exposed Area in Bangladesh. *Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering,* 38**,** 87 - 113.

CHRISTENSEN, O. B. & LAGESSON, V. 1981. Nickel concentration of blood and urine after oral administration. *Ann Clin Lab Sci,* 11**,** 119-25.

CIARDULLO, S., AURELI, F., RAGGI, A. & CUBADDA, F. 2010. Arsenic speciation in freshwater fish: focus on extraction and mass balance. *Talanta,* 81**,** 213-21.

CLELAND, B., TSUCHIYA, A., KALMAN, D. A., DILLS, R., BURBACHER, T. M., WHITE, J. W., FAUSTMAN, E. M. & MARIEN, K. 2009. Arsenic exposure within the Korean community (United States) based on dietary behavior and arsenic levels in hair, urine, air, and water. *Environmental Health Perspectives,* 117**,** 632-638.

COMBS JR, G. F. 2001. Selenium in global food systems. *British Journal of Nutrition,* 85**,** 517-547.

COMPSTON, A., CONFAVREUX, C., LASSMANN, H., MCDONALD, I., MILLER, D. & NOSEWORTHY, J. (eds.) 2006a. *McAlpine's multiple sclerosis,* Philadelphia: Churchill Livingstone/Elsevier.

COMPSTON, A., CONFAVREUX, C., LASSMANN, H., MCDONALD, I., MILLER, D. & NOSEWORTHY, J. (eds.) 2006b. *McAlpine's multiple sclerosis,* Philadelphia: Churchill Livingstone/Elsevier.

COMPSTON, A., NOSEWORTHY, J., LASSMANN, H., MILLER, D., SMITH, K., WEKERLE, H. & CONFAVREUX, C. 2006c. *McAlpine's Multiple Sclerosis,* Philadelphia, Elsevier.

CONUNCIL OF THE EUROPEAN UNION 1998 Council Directive 98/83/EC on the quality of water intended for human consumption.

CORNELIS, R., SABBIONI, E. & VAN DER VENNE, M. T. 1994. Trace element reference values in tissues from inhabitants of the European Community. VII. Review of trace elements in blood, serum and urine of the Belgian population and critical evaluation of their possible use as reference values. *Science of the Total Environment,* 158**,** 191.

CORNS, W. T., STOCKWELL, P. B., EBDON, L. & HILL, S. J. 1993. Development of an atomic fluorescence spectrometer for the hydride-forming elements. *Journal of Analytical Atomic Spectrometry,* 8**,** 71-77.

COT 2008. COT Statement on the 2006 UK Total Diet Study of Metals and Other Elements.

CRECELIUS, E. & YAGER, J. 1997. Intercomparison of analytical methods for arsenic speciation in human urine. *Environmental Health Perspectives,* 105**,** 650-653.

CROSS, J. D., DALE, I. M. & SMITH, H. 1979. A suicide by ingestion of a mixture of copper, chromium and arsenic compounds. *Forensic Science International,* 13**,** 25-29.

CULLEN, W. R. & REIMER, K. J. 1989. Arsenic speciation in the environment. *Chemical Reviews,* 89**,** 713-764.

CUNNINGHAM, J. J., FU, A., MEARKLE, P. L. & BROWN, R. G. 1994. Hyperzincuria in individuals with insulin-dependent diabetes mellitus: Concurrent zinc status and the effect of high-dose zinc supplementation. *Metabolism: Clinical and Experimental,* 43**,** 1558-1562.

D'AMATO, M., FORTE, G. & CAROLI, S. 2004. Identification and Quantification of Major Species of Arsenic in Rice. *Journal of AOAC International,* 87**,** 238-243.

D'ILIO, S., ALESSANDRELLI, M., CRESTI, R., FORTE, G. & CAROLI, S. 2002. Arsenic content of various types of rice as determined by plasma-based techniques. *Microchemical Journal,* 73**,** 195.

DAMIANO, S., PAPETTI, P. & MENESATTI, P. 2011. Accumulation of heavy metals to assess the health status of swordfish in a comparative analysis of Mediterranean and Atlantic areas. *Mar Pollut Bull,* 62**,** 1920-5.

DAVIS, A., KEMPTON, H. & NICHOLSON, A. 1994. Groundwater transport of arsenic and chromium at a historical tannery, Woburn, Massachusetts. . *Applied Geochemistry* 9**,** 569-582.

DE BRITO-ASHURST, I., PERRY, L., SANDERS, T. A. B., THOMAS, J. E., YAQOOB, M. M. & DOBBIE, H. 2009. Dietary salt intake of Bangladeshi patients with kidney disease in East London: An exploratory case study. *e-SPEN,* 4**,** e35-e40.

DEAN A. BASS, DARRELL HICKOK, DAVID QUIG & UREK, K. 2001. Trace Element Analysis in Hair: Factors Determining Accuracy, Precision, and Reliabilit. *Alternative Medicine Review,* 6**,** 200.

DEISENHAMMER, F., BARTOS, A., EGG, R., GILHUS, N. E., GIOVANNONI, G., RAUER, S., SELLEBJERG, F. & TUMANI, T. 2011. Routine cerebrospinal fluid (CSF) analysis. *In:* GILHUS, N. E., BARNES, M. P. & BRAININ, M. (eds.) *European Handbook of Neurological Management.* Blackwell.

DEL RAZO, L. M., ARELLANO, M. A. & CEBRIÁN, M. E. 1990. The oxidation states of arsenic in well-water from a chronic arsenicism area of Northern Mexico. *Environmental Pollution,* 64**,** 143.

DELGADO, M. J., NOGALES, F., OJEDA, M. L., MURILLO, M. L. & CARRERAS, O. 2011. Effect of dietary selenite on development and intestinal absorption in offspring rats. *Life Sciences,* 88**,** 150-155.

DELLES, C., DIEZ, J. & DOMINICZAK, A. F. 2011. Urinary proteomics in cardiovascular disease: Achievements, limits and hopes. *Proteomics - Clinical Applications,* 5**,** 222-232.

DELLOW, W. J., CHAMBERS, S. T., LEVER, M., LUNT, H. & ROBSON, R. A. 1999. Elevated glycine betaine excretion in diabetes mellitus patients is associated with proximal tubular dysfunction and hyperglycemia. *Diabetes Research and Clinical Practice,* 43**,** 91.

DENKHAUS, E. & SALNIKOW, K. 2002. Nickel essentiality, toxicity, and carcinogenicity. *Critical Reviews in Oncology/Hematology,* 42**,** 35-56.

DETTWYLER, W. 1966. Some aspects of zinc metabolism in diabetics. *Diabetologia,* 2**,** 75-81.

DG ENVIRONMENT EUROPEAN COMMISSION 2000. Ambient air pollution by As, Cd and Ni compounds, position paper, working group on Arsenic, Cadmium and Nichel. Luxembourg: Office for Official Publications of the European Communities.

DIGIROLAMO, A. M. & RAMIREZ-ZEA, M. 2009. Role of zinc in maternal and child mental health. *American Journal of Clinical Nutrition,* 89.

DITTMAR, T. 2004. Hydrochemical processes controlling arsenic and heavy metal contamination in the Elqui river system (Chile). *Science of The Total Environment,* 325**,** 193.

DOBSON, A. W., ERIKSON, K. M. & ASCHNER, M. 2004. Manganese neurotoxicity.

DOPP, E., HARTMANN, L. M., VON RECKLINGHAUSEN, U., FLOREA, A. M., RABIEH, S., ZIMMERMANN, U., SHOKOUHI, B., YADAV, S., HIRNER, A. V. & RETTENMEIER, A. W. 2005. Forced uptake of trivalent and pentavalent methylated and inorganic arsenic and its cyto-/genotoxicity in fibroblasts and hepatoma cells. *Toxicological Sciences,* 87**,** 46-56.

DOPP, E., VON RECKLINGHAUSEN, U., HARTMANN, L. M., STUECKRADT, I., POLLOK, I., RABIEH, S., HAO, L., NUSSLER, A., KATIER, C., HIRNER, A. V. & RETTENMEIER, A. W. 2008. Subcellular distribution of inorganic and methylated arsenic compounds in human urothelial cells and human hepatocytes. *Drug Metabolism and Disposition,* 36**,** 971-979.

DORE-DUFFY, P., NEWMAN, W., BALABANOV, R., LISAK, R. P., MAINOLFI, E., ROTHLEIN, R. & PETERSON, M. 1995. Circulating, soluble adhesion proteins in cerebrospinal fluid and serum of patients with multiple sclerosis: Correlation with clinical activity. *Annals of Neurology,* 37**,** 55-62.

DUBITZKY, W., GRANZOW, M., BERRAR, D., BERRAR, D., GRANZOW, M. & DUBITZKY, W. 2007. Introduction to Genomic and Proteomic Data Analysis. *Fundamentals of Data Mining in Genomics and Proteomics.* Springer US.

DUMONT, E., VANHAECKE, F. & CORNELIS, R. 2006. Selenium speciation from food source to metabolites: A critical review. *Analytical and Bioanalytical Chemistry,* 385**,** 1304-1323.

DYMENT, D. A., DESSA SADNOVICH, A. & EBERS, G. C. 1997. Genetics of multiple sclerosis. *Human Molecular Genetics,* 6**,** 1693-1698.

EASTMENT, H. & KRZANOWSKI, W. 1982. Crossvalidatory choice of the number of components from a principal component analysis. *Technometrics* 24 73-77.

EFSA. 2009. Scientific opinion on Arsenic in Food. *EFSA Journal 2009* [Online], 7.

ELLIS, J. Year. Metabolic Profiling Detects Systemic Effects of Environmental and Lifestyle Exposure to Cadmium in a Human Population. *In:* Metabolomics2010, 2010 Amsterdam.

ENGELHARDT, B. & RANSOHOFF, R. M. 2005. The ins and outs of T-lymphocyte trafficking to the CNS: Anatomical sites and molecular mechanisms. *Trends in Immunology,* 26**,** 485-495.

ENGSTROM, K., VAHTER, M., MLAKAR, S. J., CONCHA, G., NERMELL, B., RAQIB, R., CARDOZO, A. & BROBERG, K. 2011. Polymorphisms in Arsenic(+III Oxidation State) Methyltransferase (AS3MT) Predict Gene Expression of AS3MT as Well as Arsenic Metabolism. *Environ Health Perspect,* 119.

ENGSTRÖM, K. S., BROBERG, K., CONCHA, G., NERMELL, B., WARHOLM, M. & VAHTER, M. 2007. Genetic polymorphisms influencing arsenic metabolism: Evidence from Argentina. *Environmental Health Perspectives,* 115**,** 599-605.

EPA. 1996. *Manganese CASRN 7439-96-5* [Online]. Available: http://www.epa.gov/NCEA/iris/subst/0373.htm#oralrfd [Accessed].

EPA 2004. Public Health Statement for Cobalt.

ESPEJO, C. & MARTÍNEZ-CÁCERES, E. M. 2005. The role of methallothioneins in experimental autoimmune encephalomyelitis and multiple sclerosis.

ETTINGER, A. S., ZOTA, A. R., AMARASIRIWARDENA, C. J., HOPKINS, M. R., SCHWARTZ, J., HU, H. & WRIGHT, R. O. 2009. Maternal Arsenic Exposure and Impaired Glucose Tolerance during Pregnancy. *Environ Health Perspect,* 117**,** 1059-1064.

EUROPEAN PARLIAMENT 2001. Clinical Trial Directive 2001/20/EC. *In:* PARLIAMENT, E. (ed.). Official Journal of the European Communities.

EVM 2003. Safe upper levels for vitamins and minerals. Report of the Expert Group on Vitamins and Mineral.

EXPERT GROUP ON VITAMINS AND MINERALS 2003. Risk Assessment Copper.

FAGOT-CAMPAGNA, A., PETTITT, D. J., ENGELGAU, M. M., RIÌOS BURROWS, N., GEISS, L. S., VALDEZ, R., BECKLES, G. L. A., SAADDINE, J., GREGG, E. W., WILLIAMSON, D. F. & VENKAT NARAYAN, K. M. 2000. Type 2 diabetes among North American children and adolescents: An epidemiologic review and a public health perspective. *Journal of Pediatrics,* 136**,** 664.

FALLICO, R. & FERRANTE, M. 1999. Dietary Intake of Some elements in the Catania Area. Part II: Chromium, Manganese, Cobalt and Nickel. *L'Igiene Moderna,* 122**,** 1961-1962.

FALLICO, R. & FERRANTE, M. 2000. Dietary Intake of some elements in the Catania Area. Part III: Lead, mercury and cadmium. *Igiene Moderna,* 113**,** 199.

FANO, V., CERNIGLIARO, A., SCONDOTTO, S., CUCCIA, M., FORASTIERE, F., NICOLOSI, A., OLIVERI, C., SCILLIERI, R., DISTEFANO, P. & PERUCCI, C. A. 2005. Health effects of environmental contamination due to volcanic ash of Mount Etna in autumn 2002. *Effetti sulla salute della popolazione derivanti dalla contaminazione amibientale delle ceneri dell'Etna nell'autunno 2002.,* 29**,** 180-187.

FANO, V., CERNIGLIARO, A., SCONDOTTO, S., PERUCCI, C. A. & FORASTIERE, F. 2010. The fear of volcano: short-term health effects after Mount Etna's eruption in 2002. *European Respiratory Journal,* 36**,** 1216-1218.

FELDMANN, J., JOHN, K. & PENGPRECHA, P. 2000. Arsenic metabolism in seaweed-eating sheep from Northern Scotland. *Fresenius J Anal Chem,* 368**,** 116-21.

FELDMANN, J., LAI, V. W.-M., CULLEN, W. R., MA, M., LU, X. & LE, X. C. 1999. Sample Preparation and Storage Can Change Arsenic Speciation in Human Urine. *Clin Chem,* 45**,** 1988-1997.

FERRANTE, M., FALLICO, R., CASCIO, C., OLIVETI CONTI, G., MICELI, G. & SCIACCA, S. Year. Risk assay of phatologies from methil-mercury and hexaclorobenzene in a high industrial density zone in Sicily *In:* 13th World Clean Air and Environmental Protection Congress, , 2004 London, UK, August 2004.

FLURI, F., LYRER, P., GRATWOHL, A., RAETZ-BRAVO, A. E. & STECK, A. J. 2007. Lead poisoning from the beauty case: Neurologic manifestations in an elderly woman. *Neurology,* 69**,** 929-930.

FOOD AND NUTRITION BOARD, I. O. M., NATIONAL ACADEMIE, 2001. Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc http://www.iom.edu/Activities/Nutrition/SummaryDRIs/DRI-Tables.aspx.

FORD, E. S. 2000. Serum copper concentration and coronary heart disease among US adults. *American Journal of Epidemiology,* 151**,** 1182-1188.

FORSBERG, A. M., NILSSON, E., WERNEMAN, J., BERGSTROM, J. & HULTMAN, E. 1991. Muscle composition in relation to age and sex. *Clinical Science,* 81**,** 249-256.

FORTE, G., BOCCA, B., SENOFONTE, O., PETRUCCI, F., BRUSA, L., STANZIONE, P., ZANNINO, S., VIOLANTE, N., ALIMONTI, A. & SANCESARIO, G. 2004. Trace and major elements in whole blood, serum, cerebrospinal fluid and urine of patients with Parkinson's disease. *Journal of Neural Transmission,* 111**,** 1031-1040.

FORTE, G., VISCONTI, A., SANTUCCI, S., GHAZARYAN, A., FIGÀ-TALAMANCA, L., CANNONI, S., BOCCA, B., PINO, A., VIOLANTE, N., ALIMONTI, A., SALVETTI, M. & RISTORI, G. 2005. Quantification of chemical elements in blood of patients affected by multiple sclerosis. *Annali dell'Istituto Superiore di Sanita,* 41**,** 213-216.

FORTI, E., SALOVAARA, S., CETIN, Y., BULGHERONI, A., TESSADRI, R., JENNINGS, P., PFALLER, W. & PRIETO, P. 2011. In vitro evaluation of the toxicity induced by nickel soluble and particulate forms in human airway epithelial cells. *Toxicology in Vitro,* 25**,** 454-461.

FOSTER, H. D. 1993. The Iodine-selenium connection: Its possible roles in intelligence, cretinism, sudden infant death syndrome, breast cancer and multiple sclerosis. *Medical Hypotheses,* 40**,** 61-65.

FRANCESCONI, K. A. 2007. Toxic metal species and food regulations - Making a healthy choice. *Analyst,* 132**,** 17-20.

FRANCESCONI, K. A. & KUEHNELT, D. 2004. Determination of arsenic species: A critical review of methods and applications, 2000-2003. *Analyst,* 129**,** 373-395.

FREEDMAN, M. S., THOMPSON, E. J., DEISENHAMMER, F., GIOVANNONI, G., GRIMSLEY, G., KEIR, G., OHMAN, S., RACKE, M. K., SHARIEF, M., SINDIC, C. J. M., SELLEBJERG, F. & TOURTELLOTTE, W. W. 2005. Recommended Standard of Cerebrospinal Fluid Analysis in the Diagnosis of Multiple Sclerosis: A Consensus Statement. *Arch Neurol,* 62**,** 865-870.

FRISBIE, S. H., MITCHELL, E. J., MASTERA, L. J., MAYNARD, D. M., YUSUF, A. Z., SIDDIQ, M. Y., ORTEGA, R., DUNN, R. K., WESTERMAN, D. S., BACQUART, T. & SARKAR, B. 2009. Public health strategies for western Bangladesh that address arsenic manganese, uranium, and other toxic elements in drinking water. *Environmental Health Perspectives,* 117**,** 410-416.

FSA 2003. COT Statement on the 2006 UK Total Diet Study of Metals and Other Elements.

FSA January 2009. MEASUREMENT OF THE CONCENTRATIONS OF METALS AND OTHER ELEMENTS FROM THE 2006 UK TOTAL DIET STUDY. *http://www.food.gov.uk/science/surveillance/*.

FU, J., WOODS, C. G., YEHUDA-SHNAIDMAN, E., ZHANG, Q., WONG, V., COLLINS, S., SUN, G., ANDERSEN, M. E. & PI, J. 2010. Low-Level Arsenic Impairs Glucose-Stimulated Insulin Secretion in Pancreatic Beta Cells: Involvement of Cellular Adaptive Response to Oxidative Stress. *Environ Health Perspect,* 118.

GAILER, J., GEORGE, G. N., PICKERING, I. J., PRINCE, R. C., RINGWALD, S. C., PEMBERTON, J. E., GLASS, R. S., YOUNIS, H. S., DEYOUNG, D. W. & APOSHIAN, H. V. 2000a. A metabolic link between arsenite and selenite: The seleno-bis(S- glutathionyl) arsinium ion. *Journal of the American Chemical Society,* 122**,** 4637-4639.

GAILER, J., MADDEN, S., BURKE, M. F., DENTON, M. B. & APOSHIAN, H. V. 2000b. Simultaneous multielement-specific detection of a novel glutathione–arsenic–selenium ion [(GS)2AsSe]− by ICP AES after micellar size- exclusion chromatography. *Applied Organometallic Chemistry,* 14**,** 355.

GAILER, J., MADDEN, S., BUTTIGIEG, G. A., BONNER DENTON, M. & YOUNIS, H. S. 2002. Identification of [(GS)2AsSe]- in rabbit bile by size-exclusion chromatography and simultaneous multielement-specific detection by inductively coupled plasma atomic emission spectroscopy. *Applied Organometallic Chemistry,* 16**,** 72-75.

GAMBLE, M. V., LIU, X., AHSAN, H., PILSNER, J. R., ILLIEVSKI, V., SLAVKOVICH, V., PARVEZ, F., LEVY, D., FACTOR-LITVAK, P. & GRAZIANO, J. H. 2005. Folate, homocysteine, and arsenic metabolism in arsenic-exposed individuals in Bangladesh. *Environmental Health Perspectives,* 113**,** 1683-1688.

GAMBLE, M. V., LIU, X., SLAVKOVICH, V., PILSNER, J. R., ILIEVSKI, V., FACTOR-LITVAK, P., LEVY, D., ALAM, , S., I., M.,, PARVEZ, F., AHSAN, H. & GRAZIANO, J. H. 2007. Folic acid supplementation lowers blood arsenic. *American Journal of Clinical Nutrition,* 86**,** 1202-1209.

GAMMELGAARD, B., BENDAHL, L., JACOBSEN, N. W. & STÜRUP, S. 2005. Quantitative determination of selenium metabolites in human urine by LC-DRC-ICP-MS. *Journal of Analytical Atomic Spectrometry,* 20**,** 889-893.

GAMMELGAARD, B., JACKSON, M. I. & GABEL-JENSEN, C. 2011. Surveying selenium speciation from soil to cell-forms and transformations. *Analytical and Bioanalytical Chemistry,* 399**,** 1743-1763.

GARLAND, M., MORRIS, J. S., ROSNER, B. A., STAMPFER, M. J., SPATE, V. L., BASKETT, C. J., WILLETT, W. C. & HUNTER, D. J. 1993. Toenail trace element levels as biomarkers: Reproducibility over a 6-year period *Cancer Epidem. Biomarkers* 2**,** 493-497.

GARNER, L. A. 2004. Contact dermatitis to metals. *Dermatol. Ther.,* 17**,** 321-327.

GARTLAND, K. P., BEDDELL, C. R., LINDON, J. C. & NICHOLSON, J. K. 1991. Application of pattern recognition methods to the analysis and classification of toxicological data derived from proton nuclear magnetic resonance spectroscopy of urine. *Mol Pharmacol,* 39**,** 629-42.

GARTLAND, K. P., BONNER, F. W. & NICHOLSON, J. K. 1989. Investigations into the biochemical effects of region-specific nephrotoxins. *Mol Pharmacol,* 35**,** 242-50.

GAULT, A. G., ROWLAND, H. A. L., CHARNOCK, J. M., WOGELIUS, R. A., GOMEZ-MORILLA, I., VONG, S., LENG, M., SAMRETH, S., SAMPSON, M. L. & POLYA, D. A. 2008. Arsenic in hair and nails of

individuals exposed to arsenic-rich groundwaters in Kandal province, Cambodia. *Science of The Total Environment,* 393**,** 168-176.

GAUTHIER, P. J. & LE CLOAREC, M. F. 1998. Variability of alkali and heavy metal fluxes released by Mt. Etna volcano, Sicily, between 1991 and 1995. *Journal of Volcanology and Geothermal Research,* 81**,** 311-326.

GELLEIN, K., SKOGHOLT, J. H., AASETH, J., THORESEN, G. B., LIERHAGEN, S., STEINNES, E., SYVERSEN, T. & FLATEN, T. P. 2008. Trace elements in cerebrospinal fluid and blood from patients with a rare progressive central and peripheral demyelinating disease. *Journal of the Neurological Sciences,* 266**,** 70-78.

GENOME CANADA & ALBERTA, G. *Human Metabolome Database: metabocard for Zinc (HMDB01303)* [Online]. Human Metabolome Project. Available: http://www.hmdb.ca/metabolites/HMDB01303 [Accessed 10/07/2011].

GERAGHTY & INC, M. (eds.) 1996. *Status report on the groundwater extraction system* Bryan, Texas.

GERHARDSSON, L., LUNDH, T., MINTHON, L. & LONDOS, E. 2008. Metal concentrations in plasma and cerebrospinal fluid in patients with Alzheimer's disease. *Dementia and Geriatric Cognitive Disorders,* 25**,** 508-515.

GHOSH, D. & POISSON, L. M. 2009. "Omics" data and levels of evidence for biomarker discovery. *Genomics,* 93**,** 13-16.

GHOSH, P., BANERJEE, M., GIRI, A. K. & RAY, K. 2008. Toxicogenomics of arsenic: Classical ideas and recent advances. *Mutation Research/Reviews in Mutation Research,* 659**,** 293-301.

GHOSH, P., BASU, A., MAHATA, J., BASU, S., SENGUPTA, M., DAS, J. K., MUKHERJEE, A., SARKAR, A. K., MONDAL, L., RAY, K. & GIRI, A. K. 2006. Cytogenetic damage and genetic variants in the individuals susceptible to arsenic-induced cancer through drinking water. *Int. J. Cancer.***,** 2470–2478.

GIAMMANCO, S., VALENZA, M., PIGNATO, S. & GIAMMANCO, G. 1996. Mg, Mn, Fe, and V concentrations in the ground waters of Mount Etna (Sicily). *Water Research,* 30**,** 378-386.

GIROUARD, E. & ZAGURY, G. J. 2009. Arsenic bioaccessibility in CCA-contaminated soils: Influence of soil properties, arsenic fractionation, and particle-size fraction. *Science of The Total Environment,* 407**,** 2576-2585.

GIUFFRIDA, M. L., GRASSO, G., RUVO, M., PEDONE, C., SAPORITO, A., MARASCO, D., PIGNATARO, B., CASCIO, C., COPANI, A. & RIZZARELLI, E. 2007. Aβ(25-35) and its C- and/or N-blocked derivatives: Copper driven structural features and neurotoxicity. *Journal of Neuroscience Research,* 85**,** 623-633.

GOEBELER, M., MEINARDUS-HAGER, G., ROTH, J., GOERDT, S. & SORG, C. 1993. Nickel chloride and cobalt chloride, two common contact sensitizers, directly induce expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule (ELAM-1) by endothelial cells. *Journal of Investigative Dermatology,* 100**,** 759-765.

GOLDSCHMIDT, V. M. 1923 Geochemische Verteilungsgesetze der Elemente. *Vidensk. skrifter. I. Mat.-naturv. klasse.,* 1.

GÓMEZ, J. J., LILLO, J. & SAHÚN, B. 2006. Naturally occurring arsenic in groundwater and identification of the geochemical sources in the Duero Cenozoic Basin, Spain. *Environmental Geology,* 50**,** 1151-1170.

GONDI, F., PANTO, G., FEHER, J., BOGYE, G. & ALFTHAN, G. 1992. Selenium in Hungary: The rock-soil-human system. *Biological Trace Element Research,* 35**,** 299.

GONG, Z., LU, X., CULLEN, W. R. & LE, X. C. 2001. Unstable trivalent arsenic metabolites, monomethylarsonous acid and dimethylarsinous acid. *Journal of Analytical Atomic Spectrometry,* 16**,** 1409-1413.

GOULLÉ, J.-P., MAHIEU, L., CASTERMANT, J., NEVEU, N., BONNEAU, L., LAINÉ, G., BOUIGE, D. & LACROIX, C. 2005. Metal and metalloid multi-elementary ICP-MS validation in whole blood, plasma, urine and hair: Reference values. *Forensic Science International,* 153**,** 39.

GRAMATICA, P., BATTAINI, F., GIANI, E., PAPA, E., JONES, R. J. A., PREATONI, D. & CENCI, R. M. 2006. Analysis of Mosses and Soils for Quantifying Heavy Metal Concentrations in Sicily: A Multivariate and Spatial Analytical Approach. *Environmental Science and Pollution Research,* 13**,** 28-36.

GRANIERI, E., CASETTA, I., GOVONI, V., TOLA, M. R., MARCHI, D., MURGIA, S. B., TICCA, A., PUGLIATTI, M., MURGIA, B. & ROSATI, G. 2000. The increasing incidence and prevalence of MS in a Sardinian province. *Neurology,* 55**,** 842-847.

GRIFFIN, J. L., WALKER, L., SHORE, R. F. & NICHOLSON, J. K. 2001. High-resolution magic angle spinning 1H-NMR spectroscopy studies on the renal biochemistry in the bank vole (Clethrionomys glareolus) and the effects of arsenic (As3+) toxicity. *Xenobiotica,* 31**,** 377-385.

GUHA MAZUMDER, D. N., CHAKRABORTY, A. K., GHOSE, A., GUPTA, J. D., CHAKRABORTY, D. P., DEY, S. B. & CHATTOPADHYAY, N. 1988. Chronic arsenic toxicity from drinking tubewell water in rural West Bengal. *Bulletin of the World Health Organization,* 66**,** 499-506.

GUHA MAZUMDER, D. N., HAQUE, R., GHOSH, N., DE, B. K., SANTRA, A., CHAKRABORTY, D. & SMITH, A. H. 1998. Arsenic levels in drinking water and the prevalence of skin lesions in West Bengal, India. *Int J Epidemiol,* 27**,** 871-7.

GUNSHIN, H., MACKENZIE, B., BERGER, U. V., GUNSHIN, Y., ROMERO, M. F., BORON, W. F., NUSSBERGER, S., GOLLAN, J. L. & HEDIGER, M. A. 1997. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature,* 388**,** 482.

GURZAU, E. S. & GURZAU, A. E. 2001. Arsenic in drinking water from groundwater in Transylvania, Romania: An overview. *Chappell, W.R., Abernathy, C.O. & Calderon, R.L., eds, Arsenic Exposure and Health Effects IV.*

HABEK, M., HOJSAK, I. & BRINAR, V. V. 2010. Nutrition in multiple sclerosis. *Clinical Neurology and Neurosurgery,* 112**,** 616-620.

HABUDA-STANIC, KULES, M., KALAJDZIC & ROMIC 2007. Quality of groundwater in eastern Croatia. The problem of arsenic pollution. *Desalination,* 210**,** 157-162.

HAFEMAN, D. M., AHSAN, H., LOUIS, E. D., SIDDIQUE, A. B., SLAVKOVICH, V., CHENG, Z., VAN GEEN, A. & GRAZIANO, J. H. 2005. Association between arsenic exposure and a measure of subclinical sensory neuropathy in Bangladesh. *Journal of Occupational and Environmental Medicine,* 47**,** 778-784.

HALL, M. N., LIU, X., SLAVKOVICH, V., ILIEVSKI, V., PILSNER, J. R., ALAM, S., FACTOR-LITVAK, P., GRAZIANO, J. H. & GAMBLE, M. V. 2009. Folate, Cobalamin, Cysteine, Homocysteine, and Arsenic Metabolism among Children in Bangladesh. *Environ Health Perspect,* 117.

HAMILTON, E. I., SABBIONI, E. & VAN DER VENNE, M. T. 1994a. Element reference values in tissues from inhabitants of the European community. VI. Review of elements in blood, plasma and urine and a critical evaluation of reference values for the United Kingdom population. *Science of The Total Environment,* 158**,** 165-190.

HAMILTON, E. I., SABBIONI, E. & VAN DER VENNE, M. T. 1994b. Element reference values in tissues from inhabitants of the European Community. VI. Review of elements in blood, plasma and urine and a critical evaluation of reference values for the United Kingdom population. *Science of the Total Environment,* 158**,** 165.

HANSELL, A. L., HORWELL, C. J. & OPPENHEIMER, C. 2006. The health hazards of volcanoes and geothermal areas. *Occupational and Environmental Medicine,* 63**,** 149-156.

HANSEN, H. R., PICKFORD, R., THOMAS-OATES, J., JASPARS, M. & FELDMANN, J. 2004. 2-Dimethylarsinothioyl Acetic Acid Identified in a Biological Sample: The First Occurrence of a Mammalian Arsinothioyl Metabolite. *Angewandte Chemie - International Edition,* 43**,** 337-340.

HANSEN, H. R., RAAB, A., PRICE, A. H., DUAN, G., ZHU, Y., NORTON, G. J., FELDMANN, J. & MEHARG, A. A. 2011. Identification of tetramethylarsonium in rice grains with elevated arsenic content. *Journal of Environmental Monitoring,* 13**,** 32-34.

HANWELL, H. E. C. & BANWELL, B. 2011. Assessment of evidence for a protective role of vitamin D in multiple sclerosis. *Biochimica et Biophysica Acta - Molecular Basis of Disease,* 1812**,** 202-212.

HAQUE, R., MAZUMDER, D. N. G., SAMANTA, S., GHOSH, N., KALMAN, D., SMITH, M. M., MITRA, S., SANTRA, A., LAHIRI, S., DAS, S., DE, B. K. & SMITH, A. H. 2003. Arsenic in drinking water and skin lesions: Dose-response data from West Bengal, India. *Epidemiology,* 14**,** 174-182.

HARLEY, M. R. 1993. Anatomy and physiology of hair. *Forensic Science International,* 63**,** 9-18.

HARVEY, C. F., SWARTZ, C. H., BADRUZZAMAN, A. B. M., KEON-BLUTE, N., YU, W., ALI, M. A., JAY, J., BECKIE, R., NIEDAN, V., BRABANDER, D., OATES, P. M., ASHFAQUE, K. N., ISLAM, S.,

HEMOND, H. F. & AHMED, M. F. 2002. Arsenic mobility and groundwater extraction in Bangladesh. *Science,* 298**,** 1602-1606.

HASEGAWA, H., SOHRIN, Y., MATSUI, M., HOJO, M. & KAWASHLMA, M. 1994. Speciation of arsenic in natural waters by solvent extraction and hydride generation atomic absorption spectrometry. *Analytical Chemistry,* 66**,** 3247-3252.

HASTIE, T., TIBSHIRANI, R. & FRIEDMAN, J. (eds.) 2002. *The elements of Statistical learning,* New York.

HATA, A., ENDO, Y., NAKAJIMA, Y., IKEBE, M., OGAWA, M., FUJITANI, N. & ENDO, G. 2007. HPLC-ICP-MS speciation analysis of arsenic in urine of Japanese subjects without occupational exposure. *Journal of Occupational Health,* 49**,** 217-223.

HAYAKAWA, T., KOBAYASHI, Y., CUI, X. & HIRANO, S. 2005. A new metabolic pathway of arsenite: arsenic–glutathione complexes are substrates for human arsenic methyltransferase Cyt19. *Archives of Toxicology,* 79**,** 183-191.

HE, M.-D., XU, S.-C., LU, Y.-H., LI, L., ZHONG, M., ZHANG, Y.-W., WANG, Y., LI, M., YANG, J., ZHANG, G.-B., YU, Z.-P. & ZHOU, Z. 2011. L-carnitine protects against nickel-induced neurotoxicity by maintaining mitochondrial function in Neuro-2a cells. *Toxicology and Applied Pharmacology,* 253**,** 38-44.

HE, Y. & ZHENG, Y. 2010. Assessment of in vivo bioaccessibility of arsenic in dietary rice by a mass balance approach. *Sci Total Environ,* 408**,** 1430-6.

HECK, J. E., GAMBLE, M. V., CHEN, Y., GRAZIANO, J. H., SLAVKOVICH, V., PARVEZ, F., BARON, J. A., HOWE, G. R. & AHSAN, H. 2007. Consumption of folate-related nutrients and metabolism of arsenic in Bangladesh. *American Journal of Clinical Nutrition,* 85**,** 1367-1374.

HECK, J. E., NIEVES, J. W., CHEN, Y., PARVEZ, F., BRANDT-RAUF, P. W., HOWE, G. R. & AHSAN, H. 2010. Protein and amino acid intakes in a rural area of Bangladesh. *Food and Nutrition Bulletin,* 31**,** 206-213.

HEDSTROM, A. K., SUNDQVIST, E., BAARNHIELM, M., NORDIN, N., HILLERT, J., KOCKUM, I., OLSSON, T. & ALFREDSSON, L. 2011. Smoking and two human leukocyte antigen genes interact to increase the risk for multiple sclerosis. *Brain,* 134**,** 653-64.

HEGEDUS, C. M., SKIBOLA, C. F., WARNER, M., SKIBOLA, D. R., ALEXANDER, D., LIM, S., DANGLEBEN, N., ZHANG, L., CLARK, M., PFEIFFER, R. M., STEINMAUS, C., SMITH, A. H., SMITH, M. T. & MOORE, L. E. 2008. Decreased Urinary Beta Defensin-1 Expression as a Biomarker of Response to Arsenic. *Toxicol. Sci.***,** kfn104.

HEITKEMPER, D. T., VELA, N. P., STEWART, K. R. & WESTPHAL, C. S. 2001. Determination of total and speciated arsenic in rice by ion chromatography and inductively coupled plasma mass spectrometry. *Journal of Analytical Atomic Spectrometry,* 16**,** 299-306.

HEITLAND, P. & KOLSTER, H. D. 2008. Fast determination of arsenic species and total arsenic in urine by HPLC-ICP-MS: Concentration ranges for unexposed german inhabitants and clinical case studies. *Journal of Analytical Toxicology,* 32**,** 308.

HEITLAND, P. & KÖSTER, H. D. 2006. Biomonitoring of 30 trace elements in urine of children and adults by ICP-MS. *Clinica Chimica Acta,* 365**,** 310-318.

HEITLAND, P. & KÖSTER, H. D. 2004. Fast, simple and reliable routine determination of 23 elements in urine by ICP-MS. *Journal of Analytical Atomic Spectrometry,* 19**,** 1552-1558.

HEM, J. D. 1992. Study and Interpretation of the Chemical Characteristics of Natural Water United States *Geological Survey Water-Supply Paper,* 2254.

HENTZE, M. W., MUCKENTHALER, M. U. & ANDREWS, N. C. 2004. Balancing acts: Molecular control of mammalian iron metabolism. *Cell,* 117**,** 285.

HOFFMAN, H., PHYLIKY, R. & FLEMING, C. 1988 Zinc-induced copper deficiency. *Gastroenterology,* 94**,** 508-12.

HOLLABAUGH, C. L. 2007. Chapter 2 Modification of Goldschmidt's geochemical classification of the elements to include arsenic, mercury, and lead as biophile elements. *Developments in Environmental Science.*

HOLMES, E., LOO, R. L., STAMLER, J., BICTASH, M., YAP, I. K. S., CHAN, Q., EBBELS, T., DE IORIO, M., BROWN, I. J., VESELKOV, K. A., DAVIGLUS, M. L., KESTELOOT, H., UESHIMA, H., ZHAO, L., NICHOLSON, J. K. & ELLIOTT, P. 2008. Human metabolic phenotype diversity and its association with diet and blood pressure. *Nature,* 453**,** 396-400.

HOOGEVEEN, E. K., KOSTENSE, P. J., BEKS, P. J., MACKAAY, A. J. C., JAKOBS, C., BOUTER, L. M., HEINE, R. J. & STEHOUWER, C. D. A. 1998. Hyperhomocysteinemia Is Associated With an Increased Risk of Cardiovascular Disease, Especially in Non–Insulin-Dependent Diabetes Mellitus : A Population-Based Study. *Arterioscler Thromb Vasc Biol,* 18**,** 133-138.

HOPENHAYN-RICH, C., BIGGS, M. L., SMITH, A. H., KALMAN, D. A. & MOORE, L. E. 1996. Methylation study of a population environmentally exposed to arsenic in drinking water. *Environmental Health Perspectives,* 104**,** 620.

HOPPS, H. C. 1977. The biologic bases for using hair and nail for analyses of trace elements. *Science of The Total Environment,* 7**,** 71-89.

HORWELL, C. J. & BAXTER, P. J. 2006. The respiratory health hazards of volcanic ash: a review for volcanic risk mitigation. *Bull Volcanol,* 69**,** 1-24.

HOTELLING, H. J. 1933. Analysis of a complex statistical variable into principal components. *Educational Psychology,* 24 417--441.

HOUSTON, D. F., ALLIS, M. E. & KOHLER, G. O. 1969. Amino acid composition of rce and rice by products. *Cereal Chem. ,* 46**,** 527-537.

HOWARD, A. G. & HUNT, L. E. 1993. Coupled photooxidation-hydride AAS detector for the HPLC of arsenic compounds. *Analytical Chemistry,* 65**,** 2995-2998.

HOZUMI, I., HASEGAWA, T., HONDA, A., OZAWA, K., HAYASHI, Y., HASHIMOTO, K., YAMADA, M., KOUMURA, A., SAKURAI, T., KIMURA, A., TANAKA, Y., SATOH, M. & INUZUKA, T. 2011. Patterns of levels of biological metals in CSF differ among neurodegenerative diseases. *Journal of the Neurological Sciences*.

HSIUNG, C.-S., ANDRADE, J. D., COSTA, R. & ASH, K. O. 1997. Minimizing interferences in the quantitative multielement analysis of trace elements in biological fluids by inductively coupled plasma mass spectrometry. *Clin Chem,* 43**,** 2303-2311.

HSU, L.-I., CHIU, A. W., PU, Y.-S., WANG, Y.-H., HUAN, S. K., HSIAO, C.-H., HSIEH, F.-I. & CHEN, C.-J. 2008. Comparative genomic hybridization study of arsenic-exposed and non-arsenic-exposed urinary transitional cell carcinoma. *Toxicology and Applied Pharmacology,* 227**,** 229.

HSUEH, Y.-M., CHIOU, H.-Y., HUANG, Y.-L., WU, W.-L., HUANG, C.-C., YANG, M.-H., LUE, L.-C., CHEN, G.-S. & CHEN, C.-J. 1997. Serum β-carotene level, arsenic methylation capability, and incidence of skin cancer. *Cancer Epidemiology Biomarkers and Prevention,,* 6**,** 589-596.

HSUEH, Y.-M., HUANG, Y.-L., HUANG, C.-C., WU, W.-L., CHEN, H.-M., YANG, M.-H., LUE, L.-C. & CHEN, C.-J. 1998. Urinary levels of inorganic and organic arsenic metabolites among residents in an arseniasis-hyperendemic area in Taiwan. *Journal of Toxicology and Environmental Health - Part A,* 54**,** 431-444.

HSUEH, Y. M., CHENG, G. S., WU, M. M., YU, H. S., KUO, T. L. & CHEN, C. J. 1995. Multiple risk factors associated with arsenic-induced skin cancer: effects of chronic liver disease and malnutritional status. *Br J Cancer,* 71**,** 109-14.

HUANG, Y.-K., PU, Y.-S., CHUNG, C.-J., SHIUE, H.-S., YANG, M.-H., CHEN, C.-J. & HSUEH, Y.-M. 2008. Plasma folate level, urinary arsenic methylation profiles, and urothelial carcinoma susceptibility. *Food and Chemical Toxicology,* 46**,** 929-938.

HUANG, Y. K., HUANG, Y. L., HSUEH, Y. M., WANG, J. T. J., YANG, M. H. & CHEN, C. J. 2009. Changes in urinary arsenic methylation profiles in a 15-year interval after cessation of arsenic ingestion in Southwest Taiwan. *Environmental Health Perspectives,* 117**,** 1860-1866.

HULTMAN, P. 2007. Immunotoxicology of Metals. *In:* NORDBERG, G. F., FOWLER, B. A., NORDBERG, M. & FRIBERG, L. T. (eds.) *Handbook of Toxicology of Metals.* Elsevier.

HUTCHINSON, M. 2010. Natalizumab therapy of multiple sclerosis. *Journal of Interferon and Cytokine Research,* 30**,** 787-789.

IARC 1990. On the evaluation of carcinogenic risks to humans. In: Chromium, nickel and welding. *In:* MONOGRAPHS, I. (ed.). Lyon.

IARC 2004. ARSENIC IN DRINKING-WATER. *http://monographs.iarc.fr/ENG/Monographs/vol84/mono84-6.pdf*.

IARC 2006. Cobalt in Hard Metals and Cobalt Sulfate, Gallium Arsenide, Indium Phosphide and Vanadium Pentoxid. *Monographs on the Evaluation of Carcinogenic Risks to Humans.* Lyon.

IOM 2001. Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc (2000). *Institute of Medicine, Food and Nutrition Board. Washington, DC: National Academy Press*.

ISLAM, K., HAQUE, A., KARIM, M. R., FAJOL, A., HOSSAIN, E., SALAM, K. A., ALI, N., SAUD, Z. A., RAHMAN, M., RAHMAN, M., SULTANA, P., HOSSAIN, M., AKHAND, A. A., MANDAL, A., MIYATAKA, H., HIMENO, S. & HOSSAIN, K. 2011. Dose-response relationship between arsenic exposure and the serum enzymes for liver function tests in the individuals exposed to arsenic: a cross sectional study in Bangladesh. *Environmental Health,* 10**,** 64.

ISLAM, L. N., NABI, A. H., RAHMAN, M. M., KHAN, M. A. & KAZI, A. I. 2004a. Association of clinical complications with nutritional status and the prevalence of leukopenia among arsenic patients in Bangladesh. *International journal of environmental research and public health [electronic resource].* 1**,** 74.

ISLAM, M. R., ISHIDA, M., ANDO, S., NISHIDA, T., YOSHIDA, N. & ARAKAWA, M. 2004b. Effect of Variety and Stage of Maturity on Nutritive Value of Whole Crop Rice, Yield, Botanical Fractions, Silage Fermentability and Chemical Composition. *Asian-Australasian Journal of Animal Sciences,* 17**,** 183-192.

ISTAT 2007. Annuario Statistico Regionale Sicilia 2007.

ISTITUTO CENTRALE DI STATISTICA 2001. 14° Censimento generale della popolazione.

ITO, K., PALMER, C. D., CORNS, W. T. & PARSONS, P. J. 2010. Determination of total arsenic in urine by hydride generation atomic fluorescence spectrometry: Evaluation of three on-line oxidation methods. *Journal of Analytical Atomic Spectrometry,* 25**,** 822-830.

IUPAC Compendium of Chemical Terminology, second edition. *http://old.iupac.org/publications/compendium/T.html*.

IUPAC (ed.) 1997 *Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"),* Oxford Blackwell Scientific Publications.

IVERSEN, B. S., MENNÃ, C., WHITE, M. A., KRISTIANSEN, J., CHRISTENSEN, J. M. & SABBIONI, E. 1998. Inductively coupled plasma mass spectrometric determination of molybdenum in urine from a Danish population. *Analyst,* 123**,** 81.

IVERSEN, B. S., SABBIONI, E., FORTANER, S., PIETRA, R. & NICOLOTTI, A. 2003. Trace element reference values in tissues from inhabitants of the EU. XII. Development of BioReVa program for statistical treatment. *The Science of The Total Environment,* 302**,** 1-12.

JANKONG, P., CHALHOUB, C., KIENZL, N., GOESSLER, W., FRANCESCONI, K. A. & VISOOTTIVISETH, P. 2007. Arsenic accumulation and speciation in freshwater fish living in arsenic-contaminated waters. *Environmental Chemistry,* 4**,** 11-17.

JANSEN, J., KARGES, W. & RINK, L. 2009. Zinc and diabetes - clinical links and molecular mechanisms. *Journal of Nutritional Biochemistry,* 20**,** 399-417.

JECFA 1982a. Evaluation of certain food additives and contaminants: twenty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives (WHO Technical Report Series, No. 683). Geneva.

JECFA 1982b. Toxicological evaluation of certain food additives (WHO Food Additives Series, No. 17). Cambridge: Cambridge University Press.

JECFA 1989. Toxicological evaluation of certain food additives and contaminants. . *In:* PRESS, C. U. (ed.) *WHO Food Additives Series, No. 24.* Cambridge.

JECFA 2005. A Model for Establishing Upper Levels of Intake for Nutrients and Related Substances Report of a Joint FAO/WHO Technical Workshop on Nutrient Risk Assessment.

JECFA October 2004 Development of a Scientific Collaboration to Create a Framework for Risk Assessment of Nutrients and Related Substances.

JOLLIFFE, I. T. 2002. *Principal Component Analysis*, Springer.

JONES, E. J. P., NADEAU, T. L., VOYTEK, M. A. & LANDA, E. R. 2006. Role of microbial iron reduction in the dissolution of iron hydroxysulfate minerals. *Journal of Geophysical Research G: Biogeosciences,* 111.

JONES, F. T. 2007a. A Broad View of Arsenic. *Poult Sci,* 86**,** 2-14.

JONES, F. T. 2007b. A broad view of arsenic. *Poultry Science,* 86**,** 2-14.

KABATA-PENDIAS, A. & PENDIAS, H. 2001. *Trace elements in soils and plants*, CRC Press.

KARAGAS, M. R., TOSTESON, T. D., BLUM, J., KLAUE, B., WEISS, J. E., STANNARD, V., SPATE, V. & MORRIS, J. S. 2000. Measurement of Low Levels of Arsenic Exposure: A Comparison of Water and Toenail Concentrations. *American Journal of Epidemiology,* 152**,** 84-90.

KARIM, M. M. 2000. Arsenic in groundwater and health problems in Bangladesh. *Water Research,* 34**,** 304-310.

KARIM, M. R., ALI, N., HOQUE, M. A., HAQUE, A., SALAM, K. A., RAHMAN, A., ISLAM, K., SAUD, Z. A., KHALEK, M. A., AKHAND, A. A., HOSSAIN, M., MANDAL, A., MIYATAKA, H., HIMENO, S. & HOSSAIN, K. 2010. Association between arsenic exposure and plasma cholinesterase activity: A population based study in Bangladesh. *Environmental Health: A Global Access Science Source,* 9.

KAVANAGH, P., E. FARAGO, M., THORNTON, I., GOESSLER, W., KUEHNELT, D., SCHLAGENHAUFEN, C. & J. IRGOLIC, K. 1998. Urinary arsenic species in Devon and Cornwall residents, UK. A pilot study[dagger]. *Analyst,* 123**,** 27.

KAWAMURA, R., IKUTA, H. & FUKUZUMI, S. 1941. Intoxication by manganese in well water. *Kitasato Arch. Exp. Med.,* 18**,** 145-169.

KAZI, T. G., AFRIDI, H. I., KAZI, N., JAMALI, M. K., ARAIN, M. B., JALBANI, N. & KANDHRO, G. A. 2008. Copper, chromium, manganese, iron, nickel, and zinc levels in biological samples of diabetes mellitus patients. *Biological Trace Element Research,* 122**,** 1.

KEIM, W. 1990. Nickel: An element with wide application in industrial homogeneous catalysis. *Angewandte Chemie - International Edition in English,* 29**,** 235-244.

KEMPSON, I. M., HENRY, D. & FRANCIS, J. 2009. Characterizing arsenic in preserved hair for assessing exposure potential and discriminating poisoning. *Journal of Synchrotron Radiation,* 16**,** 422-427.

KENYON, E. M., KLIMECKI, W. T., EL-MASRI, H., CONOLLY, R. B., CLEWELL, H. J. & BECK, B. D. 2008. How can biologically-based modeling of arsenic kinetics and dynamics inform the risk assessment process? -- A workshop review. *Toxicology and Applied Pharmacology,* 232**,** 359-368.

KIGUCHI, T., YOSHINO, Y., YUAN, B., YOSHIZAWA, S., KITAHARA, T., AKAHANE, D., GOTOH, M., KAISE, T., TOYODA, H. & OHYASHIKI, K. 2010. Speciation of arsenic trioxide penetrates into cerebrospinal fluid in patients with acute promyelocytic leukemia. *Leukemia Research,* 34**,** 403-405.

KIILERICH, S., HVID-JACOBSEN, K., VAAG, A. & SORENSEN, S. S. 1990. 65 Zinc absorption in patients with insulin-dependent diabetes mellitus assessed by whole-body counting technique. *Clinica Chimica Acta,* 189**,** 13-18.

KILE, M. L., HOFFMAN, E., HSUEH, Y. M., AFROZ, S., QUAMRUZZAMAN, Q., RAHMAN, M., MAHIUDDIN, G., RYAN, L. & CHRISTIANI, D. C. 2009. Variability in biomarkers of arsenic exposure and metabolism in adults over time. *Environmental Health Perspectives,* 117**,** 455-460.

KILE, M. L., HOUSEMAN, E. A., RODRIGUES, E., SMITH, T. J., QUAMRUZZAMAN, Q., RAHMAN, M., MAHIUDDIN, G., SU, L. & CHRISTIANI, D. C. 2005. Toenail arsenic concentrations, GSTT1 gene polymorphisms, and arsenic exposure from drinking water. *Cancer Epidemiology Biomarkers and Prevention,* 14**,** 2419-2426.

KINLAW, W. B., LEVINE, A. S. & MORLEY, J. E. 1983. Abnormal zinc metabolism in type II diabetes mellitus. *American Journal of Medicine,* 75**,** 273-277.

KIRK, J., PLUMB, J., MIRAKHUR, M. & MCQUAID, S. 2003. Tight junctional abnormality in multiple sclerosis white matter affects all calibres of vessel and is associated with blood–brain barrier leakage and active demyelination. *The Journal of Pathology,* 201**,** 319-327.

KNUTSSON, G. 2008. Hydrogeology in the Nordic countries. *Episodes,* 31**,** 148-154.

KOCH-HENRIKSEN, N. & SØRENSEN, P. S. 2010. The changing demographic pattern of multiple sclerosis epidemiology. *The Lancet Neurology,* 9**,** 520-532.

KOEHRLE, J., JAKOB, F., CONTEMPRE´, B. & DUMON, J. E. 2005. Iodothyronine deiodinases are selenoproteins contributing to systemic or local thyroid hormone homeostasis. *Endocrine Reviews,* 26**,** 944 -98.

KOHASHI, N., OKABAYASHI, T., HAMA, J. & KATORI, R. 1983. Decreased urinary taurine in essential hypertension. *Prog Clin Biol Res,* 125**,** 73-87.

KÖKSALDI, E., HACIŞEVKI, A. & TORUN, M. 2008. The levels of trace elements and electrolytes in serum and cerebrospinal fluid of patients with acute stroke. *Ankara Universitesi Eczacilik Fakultesi Dergisi,* 37**,** 111-121.

KONDAKIS, X. G., MAKRIS, N., LEOTSINIDIS, M., PRINOU, M. & PAPAPETROPOULOS, T. 1989. Possible health effects of high manganese concentration in drinking water. *Arch. Environ. Health.,* 44**,** 175-178.

KOURAS, A., KATSOYIANNIS, I. & VOUTSA, D. 2007. Distribution of arsenic in groundwater in the area of Chalkidiki, Northern Greece. *Journal of Hazardous Materials,* 147**,** 890-899.

KRAGH-HANSEN, U. 1990. Structure and ligand binding properties of human serum albumin. *Dan Med Bull,* 37**,** 57-84.

KRESIMON, GRÜTER & HIRNER 2001. HG/LT-GC/ICP-MS coupling for identification of metal(loid) species in human urine after fish consumption. *Fresenius' Journal of Analytical Chemistry,* 371**,** 586.

KROKSVEEN, A. C., OPSAHL, J. A., AYE, T. T., ULVIK, R. J. & BERVEN, F. S. 2011. Proteomics of human cerebrospinal fluid: Discovery and verification of biomarker candidates in neurodegenerative diseases using quantitative proteomics. *Journal of Proteomics,* 74**,** 371-388.

KUCERA, J., BENCKO, V., SABBIONI, E. & VAN DER VENNE, M. T. 1995. Review of trace elements in blood, serum and urine for the Czech and Slovak populations and critical evaluation of their possible use as reference values. *Science of the Total Environment,* 166**,** 211.

KUH, D. & BEN-SHLOMO, Y. 1997. A life course approach to chronic disease epidemiology. *A Life Course Approach to Chronic Disease Epidemiology*.

KURTZKE, J. F. 2005. Epidemiology and etiology of multiple sclerosis. *Physical Medicine and Rehabilitation Clinics of North America,* 16**,** 327-349.

LAOHAUDOMCHOK, W., LIN, X., HERRICK, R. F., FANG, S. C., CAVALLARI, J. M., CHRISTIANI, D. C. & WEISSKOPF, M. G. 2011. Toenail, blood, and urine as biomarkers of manganese exposure. *Journal of Occupational and Environmental Medicine*.

LAU, A. L. & FAILLA, M. L. 1984. Urinary excretion of zinc, copper and iron in the streptozotocin-diabetic rat. *Journal of Nutrition,* 114**,** 224-233.

LE, X.-C., CULLEN, W. R. & REIME, K. J. 1994. Human urinary arsenic excretion following one-time ingestion of arsenosugars present in seaweed and arsenobetaine present in crab and shrimp. . *Clin Chem,* 40**/,** 617-624

LE, X. C. & MA, M. 1997. Speciation of arsenic compounds by using ion-pair chromatography with atomic spectrometry and mass spectrometry detection. *Journal of Chromatography A,* 764**,** 55-64.

LEE, M. Y., BAE, O. N., CHUNG, S. M., KANG, K. T., LEE, J. Y. & CHUNG, J. H. 2002. Enhancement of platelet aggregation and thrombus formation by arsenic in drinking water: A contributing factor to cardiovascular disease. *Toxicology and Applied Pharmacology,* 179**,** 83-88.

LEE, S. J. & BENVENISTE, E. N. 1999. Adhesion molecule expression and regulation on cells of the central nervous system. *Journal of Neuroimmunology,* 98**,** 77-88.

LENZ, E. M., BRIGHT, J., WILSON, I. D., HUGHES, A., MORRISSON, J., LINDBERG, H. & LOCKTON, A. 2004. Metabonomics, dietary influences and cultural differences: a 1H NMR-based study of urine samples obtained from healthy British and Swedish subjects. *Journal of Pharmaceutical and Biomedical Analysis,* 36**,** 841-849.

LERMAN, S. A., CLARKSON, T. W. & GERSON, R. J. 1983. Arsenic uptake and metabolism by liver cells is dependent on arsenic oxidation state. *Chemico-Biological Interactions,* 45**,** 401-406.

LEVER, M., GEORGE, P., SLOW, S., ELMSLIE, J., SCOTT, R., RICHARDS, A., FINK, J. & CHAMBERS, S. 2009. Fibrates may Cause an Abnormal Urinary Betaine Loss Which is Associated with Elevations in Plasma Homocysteine. *Cardiovascular Drugs and Therapy,* 23**,** 395-401.

LEVINE, A. S., MCCLAIN, C. J. & HANDWERGER, B. S. 1983. Tissue zinc status of genetically diabetic and streptozotocin-induced diabetic mice. *American Journal of Clinical Nutrition,* 37**,** 382-386.

LEVY, L. S., JONES, K., COCKER, J., ASSEM, F. L. & CAPLETON, A. C. 2007. Background levels of key biomarkers of chemical exposure within the UK general population - Pilot study. *International Journal of Hygiene and Environmental Health,* 210**,** 387-391.

LI, L., EKSTRÖM, E.-C., GOESSLER, W., LÖNNERDAL, B., NERMELL, B., YUNUS, M., RAHMAN, A., EL ARIFEEN, S., PERSSON, L. Å. & VAHTER, M. 2008. Nutritional status has marginal influence on the metabolism of inorganic arsenic in pregnant Bangladeshi women. *Environmental Health Perspectives,* 116**,** 315-321.

LI, X., LI, B., XU, Y., WANG, Y., JIN, Y., ITOH, T., YOSHIDA, T. & SUN, G. 2011. Arsenic methylation capacity and its correlation with skin lesions induced by contaminated drinking water consumption in residents of chronic arsenicosis area. *Environmental Toxicology,* 26**,** 118.

LIAO, C. M., LIN, T. L., HSIEH, N. H. & CHEN, W. Y. 2010. Assessing the arsenic-contaminated rice (Oryza sativa) associated children skin lesions. *J Hazard Mater,* 176**,** 239-51.

LIAW, J., MARSHALL, G., YUAN, Y., FERRECCIO, C., STEINMAUS, C. & SMITH, A. H. 2008. Increased Childhood Liver Cancer Mortality and Arsenic in Drinking Water in Northern Chile. *Cancer Epidemiology Biomarkers & Prevention,* 17**,** 1982-1987.

LIEBSCHER, K. & SMITH, H. 1968. Essential and Nonessential Trace Elements. A Method of Determining Whether an Element is Essential or Nonessential in Human Tissue. *Health Criteria 18 - Arsenic*.

LIN, G. F., DU, H., CHEN, J. G., LU, H. C., GUO, W. C., MENG, H., ZHANG, T. B., ZHANG, X. J., LU, D. R., GOLKA, K. & SHEN, J. H. 2006. Arsenic-related skin lesions and glutathione S-transferase P1 A1578G (Ile105Val) polymorphism in two ethnic clans exposed to indoor combustion of high arsenic coal in one village. *Pharmacogenet. Genomics,* 16**,** 863–871.

LIN, S., SHI, Q., NIX, F. B., STYBLO, M., BECK, M. A., HERBIN-DAVIS, K. M., HALL, L. L., SIMEONSSON, J. B. & THOMAS, D. J. 2002. A NovelS-Adenosyl-l-methionine:Arsenic(III) Methyltransferase from Rat Liver Cytosol. *Journal of Biological Chemistry,* 277**,** 10795-10803.

LINDBERG, A.-L., EKSTRÖM, E.-C., NERMELL, B., RAHMAN, M., LÖNNERDAL, B., PERSSON, L.-A. & VAHTER, M. 2008. Gender and age differences in the metabolism of inorganic arsenic in a highly exposed population in Bangladesh. *Environmental Research,* 106**,** 110-120.

LINDBERG, A.-L., SOHEL, N., RAHMAN, M., PERSSON, L. Å. & VAHTER, M. 2009. Impact of Smoking and Chewing Tobacco on Arsenic-Induced Skin Lesions. *Environ Health Perspect,* 118**,** 536-538.

LINDBERG, A. L., GOESSLER, W., GRANDE?R, M., NERMELL, B. & VAHTER, M. 2007a. Evaluation of the three most commonly used analytical methods for determination of inorganic arsenic and its metabolites in urine. *Toxicology Letters,* 168**,** 310-318.

LINDBERG, A. L., GOESSLER, W., GURZAU, E., KOPPOVA, K., RUDNAI, P., KUMAR, R., FLETCHER, T., LEONARDI, G., SLOTOVA, K., GHEORGHIU, E. & VAHTER, M. 2006. Arsenic exposure in Hungary, Romania and Slovakia. *Journal of Environmental Monitoring,* 8**,** 203-208.

LINDBERG, A. L., KUMAR, R., GOESSLER, W., THIRUMARAN, R., GURZAU, E., KOPPOVA, K., RUDNAI, P., LEONARDI, G., FLETCHER, T. & VAHTER, M. 2007b. Metabolism of low-dose inorganic arsenic in a central European population: Influence of sex and genetic polymorphisms. *Environmental Health Perspectives,* 115**,** 1081-1086.

LINDH, U. 2005. Uptake of elements from a Biological Point of view. *Essential of Medical Geology*.

LINTSCHINGER, J., SCHRAMEL, P., HATALAK-RAUSCHER, A., WENDLER, I. & MICHALKE, B. 1998. A new method for the analysis of arsenic species in urine by using HPLC-ICP-MS. *Fresenius' Journal of Analytical Chemistry,* 362**,** 313-318.

LIU, J. & WAALKES, M. P. 2008. Liver is a target of arsenic carcinogenesis. *Toxicological Sciences,* 105**,** 24-32.

LJUNG, K., SELINUS, O., OTABBONG, E. & BERGLUND, M. 2006. Metal and arsenic distribution in soil particle sizes relevant to soil ingestion by children. *Applied Geochemistry,* 21**,** 1613-1624.

LJUNG, K. & VAHTER, M. 2007. Time to re-evaluate the guideline value for manganese in drinking water? *Environmental Health Perspectives,* 115**,** 1533-1538.

LOMBARDI-BOCCIA, G., AGUZZI, A., CAPPELLONI, M., DI LULLO, G. & LUCARINI, M. 2003. Total-diet study: Dietary intakes of macro elements and trace elements in Italy. *British Journal of Nutrition,* 90**,** 1117-1121.

LOURENÇO, A. S. T., BALDEIRAS, I., GRÃOS, M. & DUARTE, C. B. 2011. Proteomics-Based technologies in the discovery of biomarkers for multiple sclerosis in the cerebrospinal fluid. *Current Molecular Medicine,* 11**,** 326-349.

LOVELL, M. A., ROBERTSON, J. D., TEESDALE, W. J., CAMPBELL, J. L. & MARKESBERY, W. R. 1998. Copper, iron and zinc in Alzheimer's disease senile plaques. *Journal of the Neurological Sciences,* 158**,** 47-52.

LÜ, J. & JIANG, C. 2005. Selenium and cancer chemoprevention: Hypotheses integrating the actions of selenoproteins and selenium metabolites in epithelial and non-epithelial target cells. *Antioxidants and Redox Signaling,* 7**,** 1715-1727.

LUU, T. T. G., STHIANNOPKAO, S. & KIM, K.-W. 2009. Arsenic and other trace elements contamination in groundwater and a risk assessment study for the residents in the Kandal Province of Cambodia. *Environment International,* 35**,** 455-460.

MA, J. F., YAMAJI, N., MITANI, N., XU, X.-Y., SU, Y.-H., MCGRATH, S. P. & ZHAO, F.-J. 2008. Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. *Proceedings of the National Academy of Sciences,* 105**,** 9931-9935.

MADEDDU, R., FORTE, G., BOCCA, B., TOLU, P., SOTGIU, M. A., SOTGIU, G., MARCHAL, J. A., SOTGIU, S. & MONTELLA, A. 2011. Heavy Metals and Multiple Sclerosis in Sardinian Population (Italy). *Analytical Letters,* 44**,** 1699-1712.

MANDAL, B. K. & SUZUKI, K. T. 2002. Arsenic round the world: A review. *Talanta,* 58**,** 201-235.

MANDAL, B. K., SUZUKI, K. T. & ANZAI, K. 2007. Impact of arsenic in foodstuffs on the people living in the arsenic-affected areas of West Bengal, India. *Joutnal of Environmental Science and Health PART A,* 42**,** 1741-1752.

MARRIE, R. A. 2004. Environmental risk factors in multiple sclerosis aetiology. *Lancet Neurology,* 3**,** 709-718.

MATSUDA, M., TSUKADA, N., MIYAGI, K. & YANAGISAWA, N. 1995. Increased levels of soluble vascular cell adhesion molecule-1 (VCAM-1) in the cerebrospinal fluid and sera of patients with multiple sclerosis and human T lymphotropic virus type-1-associating myelopathy. *Journal of Neuroimmunology,* 59**,** 35-40.

MAYERSOHN, M., CONRAD, K. A. & ACHARI, R. 1983. The influence of a cooked meat meal on creatinine plasma concentration and creatinine clearance. *British Journal of Clinical Pharmacology,* 15**,** 227-230.

MBOTAKE, I. T. 2006. A preliminary study of sources of arsenic contamination in southwest Cameroon. *Journal of Environmental Hydrology,* 14**,** 1.

MCCARTY, K. M., HOUSEMAN, E. A., QUAMRUZZAMAN, Q., RAHMAN, M., MAHIUDDIN, G., SMITH, T., RYAN, L. & CHRISTIANI, D. C. 2005. The Impact of Diet and Betel Nut Use on Skin Lesions Associated with Drinking-Water Arsenic in Pabna, Bangladesh. *Environ Health Perspect,* 114.

MCCARTY, K. M., RYAN, L., HOUSEMAN, E. A., WILLIAMS, P. L., MILLER, D. P., QUAMRUZZAMAN, Q., RAHMAN, M. M., MAHIUDDIN, G., SMITH, T., GONZALEZ, E., SU, L. & CHRISTIANI, D. C. 2006. A case-control study of GST polymorphisms and arsenic related skin lesions. *Environ. Health,* 6**,** 5–14.

MCCLATCHEY, K. D. 1994. *Clinical laboratory medicine,* London, Williams & Wilkins.

MCDONALD, W. I., COMPSTON, A., EDAN, G., GOODKIN, D., HARTUNG, H. P., LUBLIN, F. D., MCFARLAND, H. F., PATY, D. W., POLMAN, C. H., REINGOLD, S. C., SANDBERG-WOLLHEIM, M., SIBLEY, W., THOMPSON, A., VAN DEN NOORT, S., WEINSHENKER, B. Y. & WOLINSKY, J. S. 2001. Recommended diagnostic criteria for multiple sclerosis: Guidelines from the International Panel on the Diagnosis of Multiple Sclerosis. *Annals of Neurology,* 50**,** 121-127.

MCDONNELL, G. V., MCMILLAN, S. A., DOUGLAS, J. P., DROOGAN, A. G. & HAWKINS, S. A. 1998. Raised CSF levels of soluble adhesion molecules across the clinical spectrum of multiple sclerosis. *Journal of Neuroimmunology,* 85**,** 186-192.

MCDOWELL, T. Y., AMR, S., LANGENBERG, P., ROYAL, W., BEVER, C., CULPEPPER, W. J. & BRADHAM, D. D. 2010. Time of birth, residential solar radiation and age at onset of multiple sclerosis. *Neuroepidemiology,* 34**,** 238-244.

MCGREGOR, D. O., DELLOW, W. J., LEVER, M., GEORGE, P. M., ROBSON, R. A. & CHAMBERS, S. T. 2001. Dimethylglycine accumulates in uremia and predicts elevated plasma homocysteine concentrations. *Kidney Int,* 59**,** 2267.

MCKEIGUE, P. M., SHAH, B. & MARMOT, M. G. 1991. Relation of central obesity and insulin resistance with high diabetes prevalence and cardiovascular risk in South Asians. *Lancet,* 337**,** 382.

MCNAIR, P., KIILERICH, S. & CHRISTIANSEN, C. 1981. Hyperzincuria in insulin treated diabetes mellitus - its relation to glucose homeostasis and insulin administration. *Clinica Chimica Acta,* 112**,** 343-348.

MEHARG, A. A. 2007. Levels of arsenic in rice - literature review. *Food Standard Agency - literature review*.

MEHARG, A. A., DEACON, C., CAMPBELL, R. C. J., CAREY, A. M., WILLIAMS, P. N., FELDMANN, J. & RAAB, A. 2008. Inorganic arsenic levels in rice milk exceed EU and US drinking water standards. *Journal of Environmental Monitoring,* 10**,** 428-431.

MEHARG, A. A. & RAAB, A. 2010. Getting to the bottom of arsenic standards and guidelines. *Environmental Science and Technology,* 44**,** 4395-4399.

MEHARG, A. A., WILLIAMS, P. N., ADOMAKO, E., LAWGALI, Y. Y., DEACON, C., VILLADA, A., CAMBELL, R. C., SUN, G., ZHU, Y. G., FELDMANN, J., RAAB, A., ZHAO, F. J., ISLAM, R., HOSSAIN, S. & YANAI, J. 2009a. Geographical variation in total and inorganic arsenic content of polished (white) rice. *Environ Sci Technol,* 43**,** 1612-7.

MEHARG, A. A., WILLIAMS, P. N., ADOMAKO, E., LAWGALI, Y. Y., DEACON, C., VILLADA, A., CAMBELL, R. C. J., SUN, G., ZHU, Y. G., FELDMANN, J., RAAB, A., ZHAO, F. J., ISLAM, R., HOSSAIN, S. & YANAI, J. 2009b. Geographical variation in total and inorganic arsenic content of polished (white) rice. *Environmental Science and Technology,* 43**,** 1612-1617.

MEHARG, A. A., WILLIAMS, P. N., ADOMAKO, E., LAWGALI, Y. Y., DEACON, C., VILLADA, A., CAMBELL, R. C. J., SUN, G., ZHU, Y. G., FELDMANN, J., RAAB, A., ZHAO, F. J., ISLAM, R., HOSSAIN, S. & YANAI, J. 2009c. Geographical Variation in Total and Inorganic Arsenic Content of Polished (White) Rice. *Environmental Science & Technology,* 43**,** 1612-1617.

MELO, T. M., LARSEN, C., WHITE, L. R., AASLY, J., SJÃ¸BAKK, T. E., FLATEN, T. P., SONNEWALD, U. & SYVERSEN, T. 2003. Manganese, copper, and zinc in cerebrospinal fluid from patients with multiple sclerosis. *Biological Trace Element Research,* 93**,** 1.

MESSANA, I., FORNI, F., FERRARI, F., ROSSI, C., GIARDINA, B. & ZUPPI, C. 1998. Proton nuclear magnetic resonance spectral profiles of urine in type II diabetic patients. *Clin Chem,* 44**,** 1529-1534.

MESTER, Z. & PAWLISZYN, J. 2000. Speciation of dimethylarsinic acid and monomethylarsonic acid by solid- phase microextraction-gas chromatography-ion trap mass spectrometry. *Journal of Chromatography A,* 873**,** 129-135.

MEZA, M. M., KOPPLIN, M. J., BURGESS, J. L. & GANDOLFI, A. J. 2004. Arsenic drinking water exposure and urinary excretion among adults in the Yaqui Valley, Sonora, Mexico. *Environmental Research,* 96**,** 119-126.

MICHALKE, B. & NISCHWITZ, V. 2010. Review on metal speciation analysis in cerebrospinal fluid-current methods and results: A review. *Analytica Chimica Acta,* 682**,** 23-36.

MIHUCZ, V. G., TATÁR, E., VIRÁG, I., ZANG, C., JAO, Y. & ZÁRAY, G. 2007. Arsenic removal from rice by washing and cooking with water. *Food Chemistry,* 105**,** 1718-1725.

MILLWARD, D. J. 1999. The nutritional value of plant-based diets in relation to human amino acid and protein requirements. *Proc Nutr Soc,* 58**,** 249-60.

MILLWARD, D. J. & GARNETT, T. 2010. Conference on 'Over- and undernutrition: challenges and approaches' Plenary Lecture 3 Food and the planet: nutritional dilemmas of reenhouse gas emission reductions through reduced intakes of meat and dairy foods. *Proceedings of the Nutrition Society,* 69**,** 103-118.

MILLWARD, D. J. & JACKSON, A. A. 2004. Protein/energy ratios of current diets in developed and developing countries compared with a safe protein/energy ratio: implications for recommended protein and amino acid intakes. *Public Health Nutrition,* 7**,** 387-405.

MILO, R. & KAHANA, E. 2010. Multiple sclerosis: Geoepidemiology, genetics and the environment. *Autoimmunity Reviews,* 9**,** A387-A394.

MILTON, A. H. & RAHMAN, M. 2002. Respiratory effects and arsenic contaminated well water in Bangladesh. *International Journal of Environmental Health Research,* 12**,** 175-179.

MILTON, A. H., SMITH, W., RAHMAN, B., HASAN, Z., KULSUM, U., DEAR, K., RAKIBUDDIN, M. & ALI, A. 2005. Chronic arsenic exposure and adverse pregnancy outcomes in Bangladesh. *Epidemiology,* 16**,** 82-86.

MINAMI, A., TAKEDA, A., NISHIBABA, D., TAKEFUTA, S. & OKU, N. 2001. Cadmium toxicity in synaptic neurotransmission in the brain. *Brain Research,* 894**,** 336.

MINOIA, C., SABBIONI, E., APOSTOLI, PIETRA, POZZOLI, GALLORINI, NICOLAOU, G., ALESSIO, L. & CAPODAGLIO, E. 1990. Trace element reference values in tissues from inhabitants of the European Community. I. A study of 46 elements in urine, blood and serum of italian subjects. *Science of the Total Environment,* 95**,** 89-105.

MINOIA, C., SABBIONI, E., RONCHI, A., GATTI, A., PIETRA, R., NICOLOTTI, A., FORTANER, S., BALDUCCI, C., FONTE, A. & ROGGI, C. 1994. Trace element reference values in tissues from inhabitants of the European community. IV. Influence of dietary factors. *Science of The Total Environment,* 141**,** 181-195.

MITRA, S. R., GUHA MAZUMDER, D. N., BASU, A., BLOCK, G., HAQUE, R., SAMANTA, S., GHOSH, N., HIRA SMITH, M. M., VON EHRENSTEIN, O. S. & SMITH, A. H. 2004. Nutritional factors and susceptibility to arsenic-caused skin lesions in West Bengal, India. *Environmental Health Perspectives,* 112**,** 1104-1109.

MOCCHEGIANI, E., MALAVOLTA, M., LATTANZIO, F., PIACENZA, F., BASSO, A., ABBATECOLA, A. M., RUSSO, A., GIOVANNINI, S., CAPOLUONGO, E., BUSTACCHINI, S., GUFFANTI, E. E., BERNABEI, R. & LANDI, F. 2011. Cu to Zn ratio, physical function, disability, and mortality risk in older elderly (ilSIRENTE study). *Age***,** 1.

MOESGAARD, S. & MORRILL, R. 2001. The need for speciation to realize the potential of selenium in disease prevention. *Trace Element Speciation for Environment, Food and Health***,** 261.

MONDAL, D., BANERJEE, M., KUNDU, M., BANERJEE, N., BHATTACHARYA, U., GIRI, A. K., GANGULI, B., SEN ROY, S. & POLYA, D. A. 2010. Comparison of drinking water, raw rice and cooking of rice as arsenic exposure routes in three contrasting areas of West Bengal, India. *Environmental Geochemistry and Health,* 6**,** 1-15.

MONDAL, D. & POLYA, D. A. 2008. Rice is a major exposure route for arsenic in Chakdaha block, Nadia district, West Bengal, India: A probabilistic risk assessment. *Applied Geochemistry,* 23**,** 2986-2997.

MOORE, K. L., SCHRÖDER, M., WU, Z.-C., MARTIN, B. G. H., HAWES, C. R., MCGRATH, S. P., HAWKESFORD, M. J., MA, J. F., ZHAO, F. J. & GROVENOR, C. R. M. 2011. High Resolution Secondary Ion Mass Spectrometry Reveals the Contrasting Subcellular Distribution of Arsenic and Silicon in Rice Roots. *Plant Physiology*.

MORTON, J. & LEESE, E. 2011. Arsenic speciation in clinical samples: Urine analysis using fast micro-liquid chromatography ICP-MS. *Analytical and Bioanalytical Chemistry,* 399**,** 1781-1788.

MORTON, J. & MASON, H. 2006. Speciation of arsenic compounds in urine from occupationally unexposed and exposed persons in the U.K. using a routine LC-ICP-MS method. *Journal of Analytical Toxicology,* 30**,** 293.

MULTIPLE SCLEROSIS SOCIETY. 2008. Available: http://www.mult-sclerosis.org/relapsingremittingmultiplesclerosis.html [Accessed].

NAKAHARA, H., YANOKURA, M. & MURAKAMI, Y. 1978. Environmental effects of geothermal waste water on the near-by river system. *Journal of Radioanalytical and Nuclear Chemistry,* 45**,** 25-36.

NAVAS-ACIEN, A., FRANCESCONI, K. A., SILBERGELD, E. K. & GUALLAR, E. Year. Seafood intake and urine concentrations of total arsenic, dimethylarsinate and arsenobetaine in the U.S. population. *In:* 3rd International Arsenic Meeting - Taiwan, 2010.

NAVAS-ACIEN, A. & GUALLAR, E. 2008. Measuring Arsenic Exposure, Metabolism, and Biological Effects: The Role of Urine Proteomics. *Toxicol. Sci.,* 106**,** 1-4.

NAVAS-ACIEN, A., SILBERGELD, E. K., PASTOR-BARRIUSO, R. & GUALLAR, E. 2008. Arsenic Exposure and Prevalence of Type 2 Diabetes in US Adults. *JAMA: The Journal of the American Medical Association,* 300**,** 814-822.

NERMELL, B., LINDBERG, A., RAHMAN, M., BERGLUND, M., PERSSON, L., ARIFEEN, S. & VAHTER, M. 2008a. Urinary arsenic concentration adjustment factors and malnutrition. *Environmental Research,* 106**,** 212 - 218.

NERMELL, B., LINDBERG, A. L., RAHMAN, M., BERGLUND, M., A?KE PERSSON, L., EL ARIFEEN, S. & VAHTER, M. 2008b. Urinary arsenic concentration adjustment factors and malnutrition. *Environmental Research,* 106**,** 212-218.

NERMELL, B., LINDBERG, A. L., RAHMAN, M., BERGLUND, M., PERSSON, L. A., ARIFEEN, S. E. & VAHTER, M. 2008c. Urinary arsenic concentration adjustment factors and malnutrition. *Environmental Research,* 106**,** 212-218.

NEWCOMBE, C., RAAB, A., WILLIAMS, P. N., DEACON, C., HARIS, P. I., MEHARG, A. A. & FELDMANN, J. 2010. Accumulation or production of arsenobetaine in humans? *Journal of Environmental Monitoring,* 12**,** 832.

NG'WALALI, P. M., KOREEDA, A., KIBAYASHI, K. & TSUNENARI, S. 1999. Fatalities by inhalation of volcanic gas at Mt. Aso crater in Kumamoto, Japan. *Legal Medicine,* 1**,** 180-184.

NHS 2004. SERIES HS NO. 14 Health Survey for England 2004 Volume 2 Methodology and documentation.

NHS 2006. Health Survey for England 2004: health of minorities-full report. *http://www.ic.nhs.uk/pubs/hse04ethnic*.

NICKSON, R., MCARTHUR, J., BURGESS, W., MATIN AHMED, K., RAVENSCROFT, P. & RAHMAN, M. 1998. Arsenic poisoning of Bangladesh groundwater [7]. *Nature,* 395**,** 338.

NICKSON, R. T., MCARTHUR, J. M., RAVENSCROFT, P., BURGESS, W. G. & AHMED, K. M. 2000. Mechanism of arsenic release to groundwater, Bangladesh and West Bengal. *Applied Geochemistry,* 15**,** 403-413.

NICOLETTI, A., LO BARTOLO, M. L., LO FERMO, S., COCUZZA, V., PANETTA, M. R., MARLETTA, C., CIANCIO, M. R., CATALDI, M. L., PATTI, F. & REGGIO, A. 2001. Prevelence and incidence of multiple sclerosis in Catania, Sicily. *Neurology,* 56**,** 62-66.

NICOLETTI, A., LO FERMO, S., REGGIO, E., TARANTELLO, R., LIBERTO, A., LE PIRA, F., PATTI, F. & REGGIO, A. 2005. A possible spatial and temporal cluster of multiple sclerosis in the town of Linguaglossa, Sicily. *J Neurol,* 252**,** 921-5.

NICOLETTI, A., PATTI, F., LO FERMO, S., MESSINA, S., BRUNO, E., RACITI, L. & ZAPPIA, M. 2009. A possible spatial and temporal cluster of multiple sclerosis in the town of Linguaglossa, Sicily: an update. *Mult Scler,* 15**,** 129-30.

NISCHWITZ, V., BERTHELE, A. & MICHALKE, B. 2008. Speciation analysis of selected metals and determination of their total contents in paired serum and cerebrospinal fluid samples: An approach to investigate the permeability of the human blood-cerebrospinal fluid-barrier. *Analytica Chimica Acta,* 627**,** 258-269.

NRC. 1999. Arsenic in Drinking Water. *Arsenic in Drinking Water* [Online].

NRC, U. 2001. Arsenic in Drinking Water: 2001 Update. *http://books.nap.edu/openbook.php?record_id=10194&page=1*.

NUNEZ-DE LA MORA, A., CHATTERTON, R. T., CHOUDHURY, O. A., NAPOLITANO, D. A. & BENTLEY, G. R. 2007. Childhood conditions influence adult progesterone levels. *PLoS Medicine,* 4**,** 0813-0821.

OBEID, O. A., MANNAN, N., PERRY, G., ILES, R. A. & BOUCHER, B. J. 1998. Homocysteine and folate in healthy east London Bangladeshis. *Lancet,* 352**,** 1829.

OFFICE FO NATIONAL STATISTICS 2011. Population Estimates by Ethnic Group 2002 – 2009.

OLIVER, S. G., WINSON, M. K., KELL, D. B. & BAGANZ, F. 1998. Systematic functional analysis of the yeast genome. *Trends in Biotechnology,* 16**,** 373.

PAI, L. H. & PRASAD, A. S. 1988. Cellular zinc in patients with diabetes mellitus. *Nutrition Research,* 8**,** 889-897.

PAIS, I. & JONES, J. B. 1997. *The handbook of trace elements*, St. Lucie Press.

PAIVA, L., MARTÍNEZ, V., CREUS, A., QUINTEROS, D. & MARCOS, R. 2008. Evaluation of micronucleus frequencies in blood lymphocytes from smelting plant workers exposed to arsenic *Environmental and Molecular Mutagenesis,* 49 200-205.

PARK, R. M., BOAL, W. L. & KREBS, J. M. 2002. Multiple sclerosis (MS) in an Illinois community. *Archives of environmental health,* 57.

PAZIRANDEH, A., BRATI, A. H. & MARAGEH, M. G. 1998. Determination of arsenic in hair using neutron activation. *Applied Radiation and Isotopes,* 49**,** 753-759.

PEARSON, G. F., GREENWAY, G. M., BRIMA, E. I. & HARIS, P. I. 2007. Rapid arsenic speciation using ion pair LC-ICPMS with a monolithic silica column reveals increased urinary DMA excretion after ingestion of rice. *Journal of Analytical Atomic Spectrometry,* 22**,** 361.

PEARSON, K. 1901. On lines and planes of closest fit to systems of points in space. . *Philosophical Magazine,* 6**,** 559-572.

PEDERSEN, S. N. & FRANCESCONI, K. A. 2000. Liquid chromatography electrospray mass spectrometry with variable fragmentor voltages gives simultaneous elemental and molecular detection of arsenic compounds. *Rapid Communications in Mass Spectrometry,* 14**,** 641-645.

PELLEGRITI, G., DE VATHAIRE, F., SCOLLO, C., ATTARD, M., GIORDANO, C., ARENA, S., DARDANONI, G., FRASCA, F., MALANDRINO, P., VERMIGLIO, F., PREVITERA, D. M., D'AZZ, G., TRIMARCHI, F. & VIGNERI, R. 2009. Papillary thyroid cancer incidence in the volcanic area of sicily. *Journal of the National Cancer Institute,* 101**,** 1575-1583.

PERRON, H. & LANG, A. 2010. The human endogenous retrovirus link between genes and environment in multiple sclerosis and in multifactorial diseases associating neuroinflammation. *Clinical Reviews in Allergy and Immunology,* 39**,** 51-61.

PIERCE, B. L., ARGOS, M., CHEN, Y., MELKONIAN, S., PARVEZ, F., ISLAM, T., AHMED, A., HASAN, R., RATHOUZ, P. J. & AHSAN, H. 2011. Arsenic exposure, dietary patterns, and skin lesion risk in Bangladesh: A prospective study. *American Journal of Epidemiology,* 173**,** 345.

PILSNER, J. R., LIU, X., AHSAN, H., ILIEVSKI, V., SLAVKOVICH, V., LEVY, D., FACTOR-LITVAK, P., GRAZIANO, J. H. & GAMBLE, M. V. 2009. Folate deficiency, hyperhomocysteinemia, low university creatinine, and hypomethylation of leukocyte DNA are risk factors for arsenic-induced skin lesions. *Environmental Health Perspectives,* 117 254-260.

PLUMB, J., MCQUAID, S., MIRAKHUR, M. & KIRK, J. 2002. Abnormal Endothelial Tight Junctions in Active Lesions and Normal-appearing White Matter in Multiple Sclerosis. *Brain Pathology,* 12**,** 154-169.

POLIZZI, S., PIRA, E., FERRARA, M., BUGIANI, M., PAPALEO, A., ALBERA, R. & PALMI, S. 2002. Neurotoxic Effects of Aluminium Among Foundry Workers and Alzheimer's Disease. *NeuroToxicology,* 23**,** 761.

POLYA, D. A., GAULT, A. G., DIEBE, N., FELDMAN, P., ROSENBOOM, J. W., GILLIGAN, E., FREDERICKS, D., MILTON, A. H., SAMPSON, M., ROWLAND, H. A. L., LYTHGOE, P. R., JONES, J. C., MIDDLETON, C. & COOKE, D. A. 2005. Arsenic hazard in shallow Cambodian groundwaters. *Mineralogical Magazine,* 69**,** 807-823.

PORTER, K. G., MCMASTER. D., ELMES, M. E. & AL., E. 1977. Anemias and low serum-copper during zinc therapy. . *Lancet. ,* 2**,** 744.

POULSEN, O. M., MOLIN CHRISTENSEN, J., SABBIONI, E. & VAN DER VENNE, M. T. 1994. Trace element reference values in tissues from inhabitants of the European Community. V. Review of trace elements in blood, serum and urine and critical evaluation of reference values for the Danish population. *Science of the Total Environment,* 141**,** 197.

POUSCHAT, P. & ZAGURY, G. J. 2006. In vitro gastrointestinal bioavailability of arsenic in soils collected near CCA-treated utility poles. *Environmental Science and Technology,* 40**,** 4317-4323.

PRASAD, A. S., BREWER, G. J., SCHOOMAKER, E. B. & AL., E. 1978. Hypocupremia induced by zinc therapy in adults. . *J. Amer. Med. Assoc.,* 240**,** 2166-2168.

PRIMATESTA, P. & BROOKES, M. 1999. Cardiovascular disease: prevalence and risk factors -Health Survey for England - The Health of Minority Ethnic Groups '99.

PSIHOGIOS, N. G., GAZI, I. F., ELISAF, M. S., SEFERIADIS, K. I. & BAIRAKTARI, E. T. 2008. Gender-related and age-related urinalysis of healthy subjects by NMR-based metabonomics. *NMR in Biomedicine,* 21**,** 195-207.

PURDEY, M. 2004. Chronic barium intoxication disrupts sulphated proteoglycan synthesis: a hypothesis for the origins of multiple sclerosis. *Medical Hypotheses,* 62**,** 746-754.

QUAYLE, B. M., MATHER, T. A., WITT, M. L. I., MAHER, B. A., MITCHELL, R., MARTIN, R. S. & CALABRESE, S. 2010. Application and evaluation of biomagnetic and biochemical monitoring of the dispersion and deposition of volcanically-derived particles at Mt. Etna, Italy. *Journal of Volcanology and Geothermal Research,* 191**,** 107-116.

RAAB, A., BASKARAN, C., FELDMANN, J. & MEHARG, A. A. 2009. Cooking rice in a high water to rice ratio reduces inorganic arsenic content. *Journal of Environmental Monitoring,* 11**,** 41.

RABER, G., KHOOMRUNG, S., TALESHI, M. S., EDMONDS, J. S. & FRANCESCONI, K. A. 2009. Identification of Arsenolipids with GC/MS. *Talanta,* 78**,** 1215-1218.

RAHMAN, M. M., CHOWDHURY, U. K., MUKHERJEE, S. C., MONDAL, B. K., PAUL, K., LODH, D., BISWAS, B. K., CHANDA, C. R., BASU, G. K., SAHA, K. C., ROY, S., DAS, R., PALIT, S. K., QUAMRUZZAMAN, Q. & CHAKRABORTI, D. 2001. Chronic arsenic toxicity in Bangladesh and West Bengal, India - A review and commentary. *Journal of Toxicology - Clinical Toxicology,* 39**,** 683-700.

RAHMAN, M. M., OWENS, G. & NAIDU, R. 2009. Arsenic levels in rice grain and assessment of daily dietary intake of As from rice in As-contaminated regions of Bangladesh. *Environmental Geochemistry and Health***,** 1-8.

RAML, R., GOESSLER, W., TRAAR, P., OCHI, T. & FRANCESCONI, K. A. 2005. Novel Thioarsenic Metabolites in Human Urine after Ingestion of an Arsenosugar, 2â€˜,3â€˜-Dihydroxypropyl 5-Deoxy-5-dimethylarsinoyl-Î²-d-riboside. *Chemical Research in Toxicology,* 18**,** 1444.

RANSOHOFF, R. M. 2007. Natalizumab for Multiple Sclerosis. *New England Journal of Medicine,* 356**,** 2622-2629.

RAVENSCROFT, P. 2007. PREDICTING THE GLOBAL EXTENT OF ARSENIC POLLUTION OF GROUNDWATER AND ITS POTENTIAL IMPACT ON HUMAN HEALTH. New York: UNICEF.

RAYMAN, M. P. 2005. Selenium in cancer prevention: a review of the evidence and mechanism of action. *Proceedings of the Nutrition Society,* 64**,** 527–542.

RAYMAN, M. P. 2008. Food-chain selenium and human health: Emphasis on intake. *British Journal of Nutrition,* 100**,** 254-268.

REGALBUTO, C., SALAMONE, S., LA ROSA, G. L., CALACIURA, F., BUSCEMA, M. & VIGNERI, R. 1998. Iodine deficiency and experience of iodine prophylaxis in Sicily. *Carenza iodica ed esperienza di iodoprofilassi in Sicilia,* 34**,** 429-436.

RIECKMANN, P., NUNKE, K., BURCHHARDT, M., ALBRECHT, M., WILTFANG, J., ULRICH, M. & FELGENHAUER, K. 1993. Soluble intercellular adhesion molecule-1 in cerebrospinal fluid: An indicator for the inflammatory impairment of the blood-cerebrospinal fluid barrier. *Journal of Neuroimmunology,* 47**,** 133-140.

RIEDER, H. P., SCHOETTLI, G. & SEILER, H. 1983. Trace Elements in Whole Blood of Multiple Sclerosis. *European Neurology,* 22**,** 85-92.

RIISE, T., NORTVEDT, M. W. & ASCHERIO, A. 2003. Smoking is a risk factor for multiple sclerosis. *Neurology,* 61**,** 1122-1124.

RISTORI, G., BRESCIANINI, S., PINO, A., VISCONTI, A., VITTORI, D., COARELLI, G., COTICHINI, R., BOCCA, B., FORTE, G., POZZILLI, C., PESTALOZZA, I., STAZI, M. A., ALIMONTI, A. & SALVETTI, M. 2011. Serum elements and oxidative status in clinically isolated syndromes: Imbalance and predictivity. *Neurology,* 76**,** 549-555.

RITSEMA, R., DUKAN, L., ROIG I NAVARRO, T., VAN LEEUWEN, W., OLIVEIRA, N., WOLFS, P. & LEBRET, E. 1998. Speciation of Arsenic Compounds in Urine by LC-ICP MS. *Applied Organometallic Chemistry,* 12**,** 591-599.

ROCCARO, P., BARONE, C., MANCINI, G. & VAGLIASINDI, F. G. A. 2007a. Removal of manganese from water supplies intended for human consumption: a case study. *Desalination,* 210**,** 205.

ROCCARO, P., BARONE, C., MANCINI, G. & VAGLIASINDI, F. G. A. 2007b. Removal of manganese from water supplies intended for human consumption: a case study. *Desalination,* 210**,** 205-214.

RODRÍGUEZ LADO, L., HENGL, T. & REUTER, H. I. 2008. Heavy metals in European soils: A geostatistical analysis of the FOREGS Geochemical database. *Geoderma,* 148**,** 189-199.

RODUSHKIN, I., ÖDMAN, F. & APPELBLAD, P. K. 1999. Multielement Determination and Lead Isotope Ratio Measurement in Alcoholic Beverages by High-Resolution Inductively Coupled Plasma Mass Spectrometry. *Journal of Food Composition and Analysis,* 12**,** 243-257.

ROSENFIELD & BEATH 1964. *Selenium, Geobotany, Biochemistry, Toxicity and Nutrition,* New York.

ROSSMAN, T. G. 2003. Mechanism of arsenic carcinogenesis: an integrated approach. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 533**,** 37-65.

ROUESSAC, F. & ROUESSAC, A. (eds.) 2007. *Chemical Analysis*.

ROYCHOWDHURY, T., UCHINO, T., TOKUNAGA, H. & ANDO, M. 2002. Survey of arsenic in food composites from an arsenic-affected area of West Bengal, India. *Food and Chemical Toxicology,* 40**,** 1611-1621.

RUMPLER, A., EDMONDS, J. S., KATSU, M., JENSEN, K. B., GOESSLER, W., RABER, G., GUNNLAUGSDOTTIR, H. & FRANCESCONI, K. A. 2008. Arsenic-Containing Long-Chain Fatty Acids in Cod-Liver Oil: A Result of Biosynthetic Infidelity? *Angew. Chem. Int.,* 47**,** 2665 – 2667.

RYAN, D., HOLZBECHER, J. & STUART, D. 1978. Trace elements in scalp-hair of persons with multiple sclerosis and of normal individuals. *Clin Chem,* 24**,** 1996-2000.

SABBIONI, E., MINOIA, C., PIETRA, R., FORTANER, S., GALLORINI, M. & SALTELLI, A. 1992. Trace element reference values in tissues from inhabitants of the European Community. II. Examples of strategy adopted and trace element analysis of blood, lymph nodes and cerebrospinal fluid of Italian subjects. *The Science of The Total Environment,* 120**,** 39-61.

SAID, H. M., CHENOWETH, M. & DUNN, A. 1989. Metabolism of 3H- and 14C-Labeled Glutamate, Proline, and Alanine in Normal and Adrenalectomized Rats Using Different Sites of Tracer Administration and Sampling. *Metabolism: Clinical and Experimental* 38**,** 718-727.

SAMANTA, G., SHARMA, R., ROYCHOWDHURY, T., CHAKRABORTI, D. & 2004. Arsenic and other elements in hair, nails, and skin-scales of arsenic victims in West Bengal, India. *Science of The Total Environment,* 326**,** 33-47.

SANCHEZ-PENA, L. C., PETROSYAN, P., MORALES, M., GONZALEZ, N. B., GUTIEREZ-OSPINA, G., DEL RAZO, L. M. & GONSEBATT, M. E. 2010. Arsenic species, AS3MT amount, and AS3MT gen expression in different brain regions of mouse exposed to arsenite. *Environmental Research,* 110**,** 428.

SANCHEZ-RODAS, D., GEISZINGER, A., GOÌMEZ-ARIZA, J. L. & FRANCESCONI, K. A. 2002. Determination of an arsenosugar in oyster extracts by liquid chromatography-electrospray mass spectrometry and liquid chromatography-ultraviolet photo-oxidation-hydride generation atomic fluorescence spectrometry. *Analyst,* 127**,** 60.

SATTAR, N., SCHERBAKOVA, O., FORD, I., O'REILLY, D. S., STANLEY, A., FORREST, E., MACFARLANE, P. W., PACKARD, C. J., COBBE, S. M. & SHEPHERD, J. 2004. Elevated alanine aminotransferase predicts new-onset type 2 diabetes independently of classical risk factors, metabolic syndrome, and C-reactive protein in the west of Scotland coronary prevention study. *Diabetes,* 53**,** 2855-60.

SAUDE, E., ADAMKO, D., ROWE, B., MARRIE, T. & SYKES, B. 2007. Variation of metabolites in normal human urine. *Metabolomics,* 3**,** 439.

SAVETTIERI, G., RAGONESE, P., ARIDON, P. & SALEMI, G. 2001. Epidemiology of multiple sclerosis in Sicily. *Neurological Sciences,* 22**,** 175-177.

SAYRE, L., PERRY, G., ATWOOD, C. & SMITH, M. 2000. The role of metals in neurodegenerative diseases. *Cell Mol Biol (Noisy-le-grand),* 46**,** 731-41.

SCHAFFER, S. W., LOMBARDINI, J. B. & AZUMA, J. 2000. Interaction between the actions of taurine and angiotensin II. *Amino Acids,* 18**,** 305-18.

SCHEINBERG, I. H. & STERNLIEB, I. 1994. Is non-Indian childhood cirrhosis caused by excess dietary copper? *The Lancet,* 344**,** 1002-1004.

SCHIFFER, R. B., MCDERMOTT, M. P. & COPLEY, C. 2001a. A Multiple Sclerosis Cluster Associated with a Small, North-Central Illinois Community. *Archives of Environmental Health: An International Journal,* 56**,** 389 - 395.

SCHIFFER, R. B., MCDERMOTT, M. P. & COPLEY, C. 2001b. A multiple sclerosis cluster associated with a small, north-central Illinois community. *Archives of Environmental Health,* 56**,** 389-395.

SCHMEISSER, E., GOESSLER, W. & FRANCESCONI, K. A. 2006 Human metabolism of arsenolipids present in cod liver. *ANALYTICAL AND BIOANALYTICAL CHEMISTRY* 385**,** 367-376.

SCHMEISSER, E., RUMPLER, A., KOLLROSER, M., RECHBERGER, G., GOESSLER, W. & FRANCESCONI, K. A. 2006. Arsenic fatty acids are human urinary metabolites of arsenolipids present in cod liver. *Angewandte Chemie - International Edition,* 45**,** 150.

SCHMITT, M. T., SCHREINEMACHERS, D., WU, K., NING, Z., ZHAO, B., LE, Z. C. & MUMFORD, J. L. 2005. Human nails as a biomarker of arsenic exposure from well water in Inner Mongolia:

Comparing atomic flourescence spectrometry and neutron activation analysis. *Biomarkers,* 10 95-104.

SCHOOF, R. A., YOST, L. J., CRECELIUS, E., IRGOLIC, K., GOESSLER, W., GUO, H. R. & GREENE, H. 1998. Dietary arsenic intake in Taiwanese districts with elevated arsenic in drinking water. *Human and Ecological Risk Assessment (HERA),* 4**,** 117-135.

SCHRAMEL, P., WENDLER, I. & ANGERER, J. 1997. The determination of metals (antimony, bismuth, lead, cadmium, mercury, palladium, platinum, tellurium, thallium, tin and tungsten) in urine samples by inductively coupled plasma-mass spectrometry. *International Archives of Occupational and Environmental Health,* 69**,** 219.

SCHROEDER, H. A., BALASSA, J. J. & TIPTON, I. H. 1966. Essential trace metals in man: Manganese. A study in homeostasis. *Journal of Chronic Diseases,* 19**,** 545-571.

SENIOR, P. & BHOPAL, R. 1994. Ethnicity as a variable in epidemiological research. . *BMJ,* 309**,** 327-9.

SHAYKHUTDINOV, R., MACINNIS, G., DOWLATABADI, R., WELJIE, A. & VOGEL, H. 2009. Quantitative analysis of metabolite concentrations in human urine samples using 13C{1H} NMR spectroscopy. *Metabolomics.* .

SHEN, Q., FAN, L. & NEWBURGER, P. E. 2006. Nuclease sensitive element binding protein 1 associates with the selenocysteine insertion sequence and functions in mammalian selenoprotein translation. *Journal of Cellular Physiology,* 207**,** 775-783.

SHEN, Z.-X., CHEN, G.-Q., NI, J.-H., LI, X.-S., XIONG, S.-M., QIU, Q.-Y., ZHU, J., TANG, W., SUN, G.-L., YANG, K.-Q., CHEN, Y., ZHOU, L., FANG, Z.-W., WANG, Y.-T., MA, J., ZHANG, P., ZHANG, T.-D., CHEN, S.-J., CHEN, Z. & WANG, Z.-Y. 1997. Use of Arsenic Trioxide (As2O3) in the Treatment of Acute Promyelocytic Leukemia (APL): II. Clinical Efficacy and Pharmacokinetics in Relapsed Patients. *Blood,* 89**,** 3354-3360.

SHENKIN, A. 2008. Basics in clinical nutrition: Physiological function and deficiency states of trace elements. *e-SPEN,* 3**,** e255-e258.

SHIBATA, Y., TSUZUKU, K., KOMORI, S., UMEDZU, C., IMAI, H. & MORITA, M. 2005. Analysis of diphenylarsinic acid in human and environmental samples by HPLC-ICP-MS. *Applied Organometallic Chemistry,* 19**,** 276-281.

SHIRAKAWA, T. & MORIMOTO, K. 1997. Interplay of cigarette smoking and occupational exposure on specific immunoglobulin E antibodies to cobalt. *Arch Environ Health,* 52**,** 124-8.

SHOCKCOR, J. P. & HOLMES, E. 2002. Metabonomic applications in toxicity screening and disease diagnosis. *Current topics in medicinal chemistry,* 2**,** 35-51.

SHOCKCOR, J. P. & HOLMES, E. 2005. Metabonomic Applications in Toxicity Screening and Disease Diagnosis. *Integrated Strategies for Drug Discovery Using Mass Spectrometry.* John Wiley & Sons, Inc.

SIGNES-PASTOR, A. J., DEACON, C., JENKINS, R. O., HARIS, P. I., CARBONELL-BARRACHINA, A. A. & MEHARG, A. A. 2009. Arsenic speciation in Japanese rice drinks and condiments. *Journal of Environmental Monitoring,* 11**,** 1930-1934.

SIGNES, A., MITRA, K., BURLÓ, F. & CARBONELL-BARRACHINA, A. A. 2008a. Effect of cooking method and rice type on arsenic concentration in cooked rice and the estimation of arsenic dietary intake in a rural village in West Bengal, India. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment,* 25**,** 1345-1352.

SIGNES, A., MITRA, K., BURLÓ, F. & CARBONELL-BARRACHINA, A. A. 2008b. Effect of two different rice dehusking procedures on total arsenic concentration in rice. *European Food Research and Technology,* 226**,** 561-567.

SIGNORELLI, S. 1997. Arsenic in volcanic gases. *Environmental Geology,* 32**,** 239-244.

SILMAN, A., LOYSEN, E., DE GRAAF, W. & SRAMEK, M. 1985. High dietary fat intake and cigarette smoking as risk factors for ischaemic heart disease in Bangladeshi male immigrants in East London. *J Epidemiol Community Health,* 39**,** 301-3.

SINGH, A. V. & ZAMBONI, P. 2009. Anomalous venous blood flow and iron deposition in multiple sclerosis. *J Cereb Blood Flow Metab,* 29**,** 1867-78.

SIROT, V., GUERIN, T., VOLATIER, J. L. & LEBLANC, J. C. 2009. Dietary exposure and biomarkers of arsenic in consumers of fish and shellfish from France. *Science of The Total Environment,* 407**,** 1875-1885.

SŁOJEWSKI, M., CZERNY, B., SAFRANOW, K., JAKUBOWSKA, K., OLSZEWSKA, M., PAWLIK, A., GOŁĄB, A., DROŹDZIK, M., CHLUBEK, D. & SIKORSKI, A. 2010. Microelements in stones, urine, and hair of stone formers: A new key to the puzzle of lithogenesis? *Biological Trace Element Research,* 137**,** 301-316.

SLOTNICK, M. J., MELIKER, J. R., KANNAN, S. & NRIAGU, J. O. 2008. Effects of nutritional measures on toenail arsenic concentration as a biomarker of arsenic exposure. *Biomarkers,* 13**,** 451-466.

SLOTNICK, M. J., MELIKER, J. R. & NRIAGU, J. O. 2007. Intra-individual variability in toenail arsenic concentrations in a Michigan population, USA. *J Expos Sci Environ Epidemiol,* 18**,** 149-157.

SLOTNICK, M. J., NRIAGU, J. O., JOHNSON, M. M., LINDER, A. M., SAVOIE, K. L., JAMIL, H. J. & HAMMAD, A. S. 2005. Profiles of trace elements in toenails of Arab-Americans in the Detroit area, Michigan. *Biological Trace Element Research,* 107**,** 113-126.

SMEESTER, L., RAGER, J. E., BAILEY, K. A., GUAN, X., SMITH, N., GARCÍA-VARGAS, G., DEL RAZO, L.-DROBNÁ, Z., KELKAR, H., STÝBLO, M. & FRY, R. C. 2011. Epigenetic Changes in Individuals with Arsenicosis. *Chemical Research in Toxicology,* 24**,** 165-167.

SMITH, D., FELDMAN, E. & FELDMAN, D. 1989. Trace element status in multiple sclerosis. *The American Journal of Clinical Nutrition,* 50**,** 136-140.

SMITH, E., NAIDU, R., ALSTON, A. M. & DONALD, L. S. 1998. Arsenic in the Soil Environment: A Review. *Advances in Agronomy.* Academic Press.

SMOLDERS, R., SCHRAMM, K.-W., NICKMILDER, M. & SCHOETERS, G. 2009. Applicability of non-invasively collected matrices for human biomonitoring. *Environmental Health,* 8**,** 8.

SNIDERMAN, A. D., BHOPAL, R., PRABHAKARAN, D., SARRAFZADEGAN, N. & TCHERNOF, A. 2007. Why might South Asians be so susceptible to central obesity and its atherogenic consequences? The adipose tissue overflow hypothesis. *International Journal of Epidemiology,* 36**,** 220-225.

SPADONI, M., VOLTAGGIO, M., CARCEA, M., CONI, E., RAGGI, A. & CUBADDA, F. 2007. Bioaccessible selenium in Italian agricultural soils: Comparison of the biogeochemical approach with a regression model based on geochemical and pedoclimatic variables. *Science of The Total Environment,* 376**,** 160-177.

SPEZIALI, M. & DI CASA, M. 2009. Copper, iron, zinc and other element concentrations in cerebrospinal fluid of Parkinson's disease patients - Considerations on literature data. *Trace Elements and Electrolytes,* 26**,** 171-176.

SQUATRITO, S., DELANGE, F. & TRIMARCHI, F. 1981. Endemic cretinism in Sicily. *Journal of Endocrinological Investigation,* 4**,** 295-302.

SRIRAMACHARI, S. & NAYAK, N. C. 2008. Indian childhood cirrhosis: Several dilemmas resolved. *Indian Journal of Medical Research,* 128**,** 93-96.

STEINMAUS, C., BATES, M. N., YUAN, Y., KALMAN, D., ATALLAH, R., REY, O. A., BIGGS, M. L., HOPENHAYN, C., MOORE, L. E., HOANG, B. K. & SMITH, A. H. 2006. Arsenic methylation and bladder cancer risk in case-control studies in Argentina and the United States. *Journal of Occupational and Environmental Medicine,* 48**,** 478-488.

STEINMAUS, C., CARRIGAN, K., KALMAN, D., ATALLAH, R., YUAN, Y. & SMITH, A. H. 2005a. Dietary Intake and Arsenic Methylation in a U.S. Population. *Environ Health Perspect,* 113.

STEINMAUS, C., YUAN, Y., KALMAN, D., ATALLAH, R. & SMITH, A. H. 2005b. Intraindividual variability in arsenic methylation in a U.S. population. *Cancer Epidemiology Biomarkers and Prevention,* 14, 919-924.

STELLA, C., BECKWITH-HALL, B., CLOAREC, O., HOLMES, E., LINDON, J. C., POWELL, J., VAN DER OUDERAA, F., BINGHAM, S., CROSS, A. J. & NICHOLSON, J. K. 2006. Susceptibility of Human Metabolic Phenotypes to Dietary Modulation. *Journal of Proteome Research,* 5**,** 2780.

SUN, G. X., WILLIAMS, P. N., ZHU, Y. G., DEACON, C., CAREY, A. M., RAAB, A., FELDMANN, J. & MEHARG, A. A. 2009. Survey of arsenic and its speciation in rice products such as breakfast cereals, rice crackers and Japanese rice condiments. *Environment International,* 35**,** 473-475.

SUZUKI, K. T., MANDAL, B., K.OGRA Y., 2002. Speciation of arsenic in body fluids. *Talanta* 58, 111-119

TAKAHASHI, Y., MINAMIKAWA, R., HATTORI, K. H., KURISHIMA, K., KIHOU, N. & YUITA, K. 2004. Arsenic Behavior in Paddy Fields during the Cycle of Flooded and Non-flooded Periods. *Environmental Science & Technology,* 38**,** 1038-1044.

TAN, L. B., CHEN, K. T., TYAN, Y. C., LIAO, P. C. & GUO, H. R. 2008. Proteomic analysis for human urinary proteins associated with arsenic intoxication. *Proteomics - Clinical Applications,* 2**,** 1087-1098.

TANNER, M. S., PORTMANN, B., MOWAT, A. P., WILLIAMS, R., PANDIT, A. N., MILLS, C. F. & BREMNER, I. 1979. Increased hepatic copper concentration in Indian childhood cirrhosis. *Lancet,* 1**,** 1203-5.

TCHOUNWOU, P. B., CENTENO, J. A. & PATLOLLA, A. K. 2004. Arsenic toxicity, mutagenesis, and carcinogenesis - A health risk assessment and management approach. *Molecular and Cellular Biochemistry,* 255 47-55.

TERENTYEVA, E. A., HAYAKAWA, K., TANAE, A., KATSUMATA, N., TANAKA, T. & HIBI, I. 1997. Urinary biotinidase and alanine excretion in patients with insulin-dependent diabetes mellitus. *European Journal of Clinical Chemistry and Clinical Biochemistry,* 35**,** 21.

TERRY, N., ZAYED, A. M., DE SOUZA, M. P. & TARUN, A. S. 2000. Selenium in higher plants. *Annual Review of Plant Biology.*

THIRUMARAN, R. K., BERMEJO, J. L., RUDNAI, P., GURZAU, E., KOPPOVA, K., GOESSLER, W., VAHTER, M., LEONARDI, G. S., CLEMENS, F., FLETCHER, T., HEMMINKI, K. & KUMAR, R. 2006. Single nucleotide polymorphisms in DNA repair genes and basal cell carcinoma of skin. *Carcinogenesis,* 27 1676–1681.

THYSSEN, J. P., MENNÉ, T. & JOHANSEN, J. D. 2009. Nickel release from inexpensive jewelry and hair clasps purchased in an EU country -- Are consumers sufficiently protected from nickel exposure? *Science of The Total Environment,* 407**,** 5315-5318.

TOKUNAGA, H., ROYCHOWDHURY, T., UCHINO, T. & ANDO, M. 2005. Urinary arsenic species in an arsenic-affected area of West Bengal, India (part III). *Applied Organometallic Chemistry,* 19**,** 246-253.

TORRES-ESCRIBANO, S., LEAL, M., VE?LEZ, D. & MONTORO, R. 2008. Total and inorganic arsenic concentrations in rice sold in Spain, effect of cooking, and risk assessments. *Environmental Science and Technology,* 42**,** 3867-3872.

TOWNSEND, A. T., MILLER, K. A., MCLEAN, S. & ALDOUS, S. 1998. The determination of copper, zinc, cadmium and lead in urine by high resolution ICP-MS. *Journal of Analytical Atomic Spectrometry,* 13**,** 1213-1219.

TRIMARCHI, F., VERMIGLIO, F., FINOCCHIARO, M. D., BATTIATO, S., LO PRESTI, V. P., LA TORRE, N., CALACIURA, F., REGALBUTO, C., SAVA, L. & VIGNERI, R. 1990. Epidemiology and clinical characteristics of endemic cretinism in Sicily. *Journal of Endocrinological Investigation,* 13**,** 543-548.

TRYGG, J. & WOLD, S. 2002. Orthogonal projections to latent structures (O-PLS). *Journal of Chemometrics,* 16**,** 119-128.

TSAI, S. Y., CHOU, H. Y., THE, H. W., CHEN, C. M. & CHEN, C. J. 2003. The effects of chronic arsenic exposure from drinking water on the neurobehavioral development in adolescence. *NeuroToxicology,* 24**,** 747-753.

TSALEV, D. L., SPERLING, M. & WELZ, B. 2000. Flow-injection hydride generation atomic absorption spectrometric study of the automated on-line pre-reduction of arsenate, methylarsonate and dimethylarsinate and high-performance liquid chromatographic separation of their -cysteine complexes. *Talanta,* 51**,** 1059-1068.

TSENG, C.-H. 2005. Blackfoot Disease and Arsenic: A Never-Ending Story. *Journal of Environmental Science and Health, Part C: Environmental Carcinogenesis and Ecotoxicology Reviews,* 23**,** 55 - 74.

TSENG, C.-H. 2009. A review on environmental factors regulating arsenic methylation in humans. *Toxicology and Applied Pharmacology,* 235**,** 338-350.

TSENG, C. H., CHONG, C. K., TSENG, C., HSUEH, Y. M., CHIOU, H. Y., TSENG, C. C. & CHEN, C. J. 2003a. Long-term arsenic exposure and ischemic heart disease in arseniasishyperendemic villages in Taiwan. *Toxicol. Lett.,* 137**,** 15-21.

TSENG, C. H., TSENG, C. P., CHIOU, H. Y., HSUEH, Y. M., CHONG, C. K. & CHEN, C. J. 2002. Epidemiologic evidence of diabetogenic effect of arsenic. *Toxicology Letters,* 133**,** 69-76.

TSENG, W. P. 1977. Effect and Dose-Response Relationship of Skin Cancer and Blackfoot disease with Arsenic. *Environ Health Perspect,* 19.

TSENG, W. P., CHU, H. M., HOW, S. W., FONG, J. M., LIN, C. S. & YEH, S. 1968a. Prevalence of skin cancer in an endemic area of chronic arsenicism in Taiwan. *Journal of the National Cancer Institute,* 40**,** 453.

TSENG, W. P., CHU, H. M., HOW, S. W., FONG, J. M., LIN, C. S. & YEH, S. 1968b. Prevalence of skin cancer in an endemic area of chronic arsenicism in Taiwan. . *Journal of the National Cancer Institute* 40 453- 463.

VEILLON, C. & PATTERSON, K. 1996. Trace elements in a commercial freeze-dried human urine reference material. *Analyst,* 121**,** 983-5.

VINCETI, M., BONVICINI, F., ROTHMAN, K. J., VESCOVI, L. & WANG, F. 2010. The relation between amyotrophic lateral sclerosis and inorganic selenium in drinking water: A population-based case-control study. *Environmental Health: A Global Access Science Source***,** 77.

VROBLESKY, D. A., LORAH, M. M. & OLIVEROS, J. P. 1989. Ground-water, surface-water, and bottom-sediment contamination in the O-Field Area, Aberdeen Proving Ground, Maryland and the possible effects of selected remedial actions on ground water. .

WALKER, A. W. (ed.) 1992. *Trace Element Analyses,* Surrey, UK.

WALTON, F. S., WATERS, S. B., JOLLEY, S. L., LECLUYSE, E. L., THOMAS, D. J. & STYBLO, M. 2003. Selenium Compounds Modulate the Activity of Recombinant Rat AsIII-Methyltransferase and the Methylation of Arsenite by Rat and Human Hepatocytes. *Chemical Research in Toxicology,* 16**,** 261.

WANG, Y., FANG, J., LEONARD, S. S. & RAO, K. M. 2004. Cadmium inhibits the electron transfer chain and induces reactive oxygen species. *Free Radic Biol Med,* 36**,** 1434-43.

WASSERMAN, G. A., LIU, X., PARVEZ, F., AHSAN, H., LEVY, D., FACTOR-LITVAK, P., KLINE, J., VAN GEEN, A., SLAVKOVICH, V., LOLACONO, N. J., CHENG, Z., ZHENG, Y. & GRAZIANO, J. H. 2006. Water manganese exposure and children's intellectual function in Araihazar, Bangladesh. *Environmental Health Perspectives,* 114**,** 124.

WASSERMAN, G. A., LIU, X., PARVEZ, F., FACTOR-LITVAK, P., AHSAN, H., LEVY, D., KLINE, J., VAN GEEN, A., MEY, J., SLAVKOVICH, V., SIDDIQUE, A. B., ISLAM, T. & GRAZIANO, J. H. 2011. Arsenic and manganese exposure and children's intellectual function. *NeuroToxicology,* 32**,** 450.

WEI, L., LIAO, P., WU, H., LI, X., PEI, F., LI, W. & WU, Y. 2009. Metabolic profiling studies on the toxicological effects of realgar in rats by 1H NMR spectroscopy. *Toxicology and Applied Pharmacology,* 234**,** 314-325.

WEISS, M., MULLER-HOCKER, J., WIEBECKE, B. & BELOHRADSKY, B. H. 1989. First Description of "Indian Childhood Cirrhosis" in a Non-Indian Infant in Europe. *Acta Pædiatrica,* 78**,** 152-156.

WELCH, A. H., WESTJOHN, D. B., HELSEL, D. R. & WANTY, R. B. 2000. Arsenic in Ground Water of the United States- Occurrence and Geochemistry. *Ground Water* 38**,** 589-604.

WEN, W., WEN, J., LU, L., LIU, H., YANG, J., CHENG, H., CHE, W., LI, L. & ZHANG, G. 2011. Metabolites of arsenic and increased DNA damage of p53 gene in arsenic plant workers. *Toxicology and Applied Pharmacology,* 254**,** 41-47.

WHITE, M. A. & SABBIONI, E. 1998. Trace element reference values in tissues from inhabitants of the European Union. X. A study of 13 elements in blood and urine of a United Kingdom population. *The Science of The Total Environment,* 216**,** 253-270.

WHO 2004. Manganese in Drinking-water http://www.who.int/water_sanitation_health/dwq/chemicals/manganese.pdf

WHO 1982. Safety evaluation of certain food additives and contaminants. *WHO Food Additives Series 17. Zinc.*

WHO 1988. Food Additive Series 24: ARSENIC. *Food Additive Series.*

WHO 1996. Trace elements in human nutrition and health.

WHO 1999. ENVIRONMENTAL HEALTH CRITERIA 210 Principles for the assessment of risks to human health from exposure to chemicals. *http://www.inchem.org/documents/ehc/ehc/ehc210.htm*.

WHO 2001. EHC 224 ARSENIC AND ARSENIC COMPOUNDS. *http://www.inchem.org/documents/ehc/ehc/ehc224.htm*.

WHO 2003. Arsenic in Drinking-water Background document for development of WHO Guidelines for Drinking-water Quality.

WHO 2006. ENVIRONMENTAL HEALTH CRITERIA 234 ELEMENTAL SPECIATION IN HUMAN HEALTH RISK ASSESSMENT. *http://www.inchem.org/pages/ehc.html*.

WIKLUND, S., JOHANSSON, E., SJOSTROM, L., MELLEROWICZ, E. J., EDLUND, U., SHOCKCOR, J. P., GOTTFRIES, J., MORITZ, T. & TRYGG, J. 2007. Visualization of GC/TOF-MS-Based Metabolomics Data for Identification of Biochemically Interesting Compounds Using OPLS Class Models. *Analytical Chemistry,* 80**,** 115-122.

WILHELM, M., EWERS, U. & SCHULZ, C. 2004. Revised and new reference values for some trace elements in blood and urine for human biomonitoring in environmental medicine. *International Journal of Hygiene and Environmental Health,* 207**,** 69.

WILLIAMS, P. N., ISLAM, M. R., ADOMAKO, E. E., RAAB, A., HOSSAIN, S. A., ZHU, Y. G., FELDMANN, J. & MEHARG, A. A. 2006. Increase in rice grain arsenic for regions of Bangladesh irrigating paddies with elevated arsenic in groundwaters. *Environmental Science & Technology,* 40**,** 4903-4908.

WILLIAMS, P. N., PRICE, A. H., RAAB, A., HOSSAIN, S. A., FELDMANN, J. & MEHARG, A. A. 2005a. Variation in arsenic speciation and concentration in paddy rice related to dietary exposure. *Environmental Science and Technology,* 39**,** 5531-5540.

WILLIAMS, P. N., PRICE, A. H., RAAB, A., HOSSAIN, S. A., FELDMANN, J. & MEHARG, A. A. 2005b. Variation in arsenic speciation and concentration in paddy rice related to dietary exposure. *Environmental Science & Technology,* 39**,** 5531-5540.

WILLIAMS, P. N., RAAB, A., FELDMANN, J. & MEHARG, A. A. 2007. Market basket survey shows elevated levels of as in South Central US processed rice compared to California: Consequences for human dietary exposure. *Environmental Science & Technology,* 41**,** 2178-2183.

WILLIAMSON, D. M. & HENRY, J. P. 2004. Challenges in addressing community concerns regarding clusters of multiple sclerosis and potential environmental exposures. *Neuroepidemiology,* 23**,** 211-216.

WINKEL, L., BERG, M., STENGEL, C. & ROSENBERG, T. 2008. Hydrogeological survey assessing arsenic and other groundwater contaminants in the lowlands of Sumatra, Indonesia. *Applied Geochemistry,* 23**,** 3019.

WINKEL, L., FELDMANN, J. & MEHARG, A. A. 2009. Quantitative and Qualitative Trapping of Volatile Methylated Selenium Species Entrained through Nitric Acid. *Environmental Science & Technology,* 44**,** 382-387.

WINKEL, L. H. E., TRANG, P. T. K., LAN, V. M., STENGEL, C., AMINI, M., HA, N. T., VIET, P. H. & BERG, M. 2011. Arsenic pollution of groundwater in Vietnam exacerbated by deep aquifer exploitation for more than a century. *Proceedings of the National Academy of Sciences,* 108**,** 1246-1251.

WÓJCIK, O. P., KOENIG, K. L., ZELENIUCH-JACQUOTTE, A., COSTA, M. & CHEN, Y. 2010. The potential protective effects of taurine on coronary heart disease. *Atherosclerosis,* 208**,** 19-25.

WOOLSON, E. A. 1983. *Emissions, cycling and effects of arsenic in soil ecosystems,* Amsterdam.

WORLD MEDICAL ASSOCIATION 2000. Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects. *JAMA.*

WUHRER, M., KOELEMAN, C. A. M. A. M. & DEELDER, A. M. 2009. Two-Dimensional HPLC Separation with Reverse-Phase-Nano-LC-MS/MS for the Characterization of Glycan Pools After Labeling with 2-Aminobenzamide. *In:* WALKER, J. M. (ed.) *Glycomics.* Humana Press.

XIE, Z. M. & HUANG, C. Y. 1998. Control of arsenic toxicity in rice plants grown on an arsenic-polluted paddy soil. *Communications in Soil Science and Plant Analysis,* 29**,** 2471-2477.

XU, J., YANG, S., CAI, S., DONG, J., LI, X. & CHEN, Z. 2010. Identification of biochemical changes in lactovegetarian urine using 1H NMR spectroscopy and pattern recognition. *Analytical and Bioanalytical Chemistry,* 396**,** 1451.

XUE, J., ZARTARIAN, V., WANG, S. W., LIU, S. V. & GEORGOPOULOS, P. 2010. Probabilistic Modeling of Dietary Arsenic Exposure and Dose and Evaluation with 2003-2004 NHANES Data. *Environ Health Perspect,* 118**,** 345-50.

YAMAMOTO, S., KONISHI, Y., MATSUDA, T., MURAI, T., SHIBATA, M. A., MATSUI-YUASA, I., OTANI, S., KURODA, K., ENDO, G. & FUKUSHIMA, S. 1995. Cancer induction by an organic arsenic

compound, dimethylarsinic acid (cacodylic acid), in F344/DuCrj rats after pretreatment with five carcinogens. *Cancer Research,* 55**,** 1271-1276.

YANG, G., ZHOU, R., YIN, S., GU, L., YAN, B., LIU, Y., LIU, Y. & LI, X. 1989. Studies of safe maximal daily dietary selenium intake in a seleniferous area in China. I. Selenium intake and tissue selenium levels of the inhabitants. *Journal of Trace Elements and Electrolytes in Health and Disease,* 3**,** 77.

YOKEL, R. A., LASLEY, S. M. & DORMAN, D. C. 2006. The speciation of metals in mammals influences their toxicokinetics and toxicodynamics and therefore human health risk assessment. *Journal of Toxicology and Environmental Health - Part B: Critical Reviews,* 9**,** 63-85.

YU, G., SUN, D. & ZHENG, Y. 2007. Health effects of exposure to natural arsenic in groundwater and coal in China: An overview of occurrence. *Environmental Health Perspectives,* 115**,** 636-642.

ZABLOTSKA, L. B., CHEN, Y., GRAZIANO, J. H., PARVEZ, F., VAN GEEN, A., HOWE, G. R. & AHSAN, H. 2008. Protective effects of B vitamins and antioxidants on the risk of arsenic-related skin lesions in Bangladesh. *Environmental Health Perspectives,* 116**,** 1056-1062.

ZAPADNIUK, B. 1992. The incidence of multiple sclerosis and the content of cobalt, boron, zinc, manganese and molybdenum in the arable soils of different climatic zones of Ukraine. *Lik Sprava,* 1**,** 111-3.

ZATTA, P., LUCCHINI, R., VAN RENSBURG, S. & TAYLOR, A. 2003. BrainRes Bull. *The role of metals in neurodegenerative processes: aluminum, manganese, and zinc.,* 62**,** 15-28.

ZEISEL, S. H. & DACOSTA, K.-A. 1986. Increase in Human Exposure to Methylamine Precursors of N-Nitrosamines after Eating Fish. *Cancer Research,* 46**,** 6136-6138.

ZENG, H., UTHUS, E. O. & COMBS JR, G. F. 2005. Mechanistic aspects of the interaction between selenium and arsenic. *Journal of Inorganic Biochemistry,* 99**,** 1269.

ZEREFOS, P. G. & VLAHOU, A. 2008. Urine Sample Preparation and Protein Profiling by Two-Dimensional Electrophoresis and Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectroscopy. *Clinical Proteomics.*

ZEREFOS, P. G., VOUGAS, K., DIMITRAKI, P., KOSSIDA, S., PETROLEKAS, A., STRAVODIMOS, K., GIANNOPOULOS, A., FOUNTOULAKIS, M. & VLAHOU, A. 2006. Characterization of the human urine proteome by preparative electrophoresis in combination with 2-DE. *Proteomics,* 6**,** 4346-4355.

ZHANG, H., CHAI, Z. & SUN, H. 2007. Human hair as a potential biomonitor for assessing persistent organic pollutants. *Environment International,* 33**,** 685-693.

ZHAO, F. J., MA, J. F., MEHARG, A. A. & MCGRATH, S. P. 2009. Arsenic uptake and metabolism in plants. *New Phytologist,* 181**,** 777-794.

ZHENG, J. & HINTELMANN, H. 2004. Hyphenation of high performance liquid chromatography with sector field inductively coupled plasma mass spectrometry for the determination of ultra-trace level anionic and cationic arsenic compounds in freshwater fish. *Journal of Analytical Atomic Spectrometry,* 19**,** 191-195.

ZHENG, W., PERRY, D. F., NELSON, D. L. & APOSHIAN, H. V. 1991. Choroid plexus protects cerebrospinal fluid against toxic metals. *The FASEB Journal,* 5**,** 2188-2193.

ZHU, Y. S., XU, H. B., HUANG, K. X., HU, W. H. & LIU, M. L. 2002. A study on human urine in a high-selenium area of China by 1H-NMR spectroscopy. *Biological Trace Element Research,* 89**,** 155.

ZIELHUIS, R. L. 1984. Recent and potential advances applicable to the protection of workers' health—biological monitoring. II. . *In:* A. BERLIN, R. E. Y. A. B. A. H. (ed.) *Assessment of toxic agents at the workplace—roles of ambient and biological monitoring.* Boston: Martinus Nijhoff

ZIEROLD, K. M., KNOBELOCH, L. & ANDERSON, H. 2004. Prevalence of chronic diseases in adults exposed to arsenic-contaminated drinking water. *American Journal of Public Health,* 94**,** 1936.